I✦I Defence Research and Recherche et développement
Development Canada pour la défense Canada

DEFENCE **R🍁D** DÉFENSE

Enzyme-linked Immunosorbant Assays for Identification of Biological Agents in Sample Unknowns: NATO SIBCA Exercise IV

H.G. Thompson and R.E. Fulton
Defence R&D Canada – Suffield

Technical Report

DRDC Suffield TR 2003-120

December 2003

Canadä

20040617 045

Enzyme-linked Immunosorbant Assays for Identification of Biological Agents in Sample Unknowns: NATO SIBCA Exercise IV
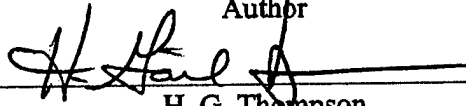
H.G. Thompson and R.E. Fulton
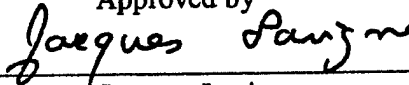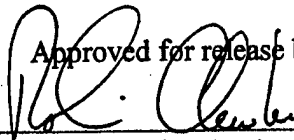Defence R&D Canada – Suffield

AQ F04-07-0389

Author

H. G. Thompson

Approved by

Dr. Jacques Lavigne

Head/Chemical and Biological Defence Section

Approved for release by

Dr. Robin Clewley

Chair DRP/DRDC Suffield

Abstract

In January 2002, the NATO Panel VII Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the fourth international training exercise on identification of biological agents. Fourteen NATO/Partners for Peace national laboratories participated: Austria, Bulgaria, Canada, Denmark, France, Germany (two laboratories), Italy, the Netherlands, Norway, Poland, Sweden, the United Kingdom, and the United States. The designated laboratory for Canada was Defence R&D Canada – Suffield (DRDC Suffield). Participant laboratories were sent six swabs. Participants were advised that samples would contain any one of the following gamma-irradiated organisms: *Bacillus anthracis, Yersinia pestis, Brucella melitensis, Francisella tularensis, Vibrio cholerae, Burkholderia mallei,* Venezuelan equine encephalitis (VEE) virus, vaccinia virus, *Coxiella burnetii,* or yellow fever virus. A number of immunologically-based technologies were used at DRDC Suffield for screening of sample unknowns, one of which was the enzyme-linked immunosorbant assay (ELISA). Antigen capture ELISAs were developed for all 10 possible biological agents and were used to screen the samples and a heterologous agent panel included as a control for specificity. Five biological agent unknowns were identified by ELISA, three at the species level: *B. melitensis, F. tularensis,* and *Y. pestis,* and two at the genus level: *Bacillus spp.* One sample containing *V. cholerae* produced a false negative reaction. A comparison of the ELISA results with the identity of organisms in SIBCA sample unknowns, as revealed by Dugway Proving Ground following the exercise, indicated correct identification of three of the samples, a partially correct identification of two samples, and an incorrect false negative for one sample.

Résumé

En janvier 2002, le Sous-groupe du panel VII de l'OTAN d'échantillonnage et d'identification des agents biologiques et chimiques (SIBCA) a conduit le quatrième exercice international de formation au sujet de l'identification des agents biologiques. Quatorze laboratoires nationaux du Partenariat pour la paix de l'OTAN ont participé : l'Autriche, la Bulgarie, le Canada, le Danemark, la France, l'Allemagne (deux laboratoires), l'Italie, les Pays-Bas, la Norvège, la Pologne, la Suède, la Grande-Bretagne et les États-Unis. Le laboratoire désigné pour le Canada était celui de R & D pour la défense, Canada – Suffield (RDDC Suffield). Les laboratoires participants ont reçu six tampons. Les participants ont été avisés que les échantillons contenaient chacun les organismes irradiés par rayons gamma suivants : *Bacillus anthracis, Yersinia pestis, Brucella melitensis, Francisella tularensis, Vibrio cholerae, Burkholderia mallei*, le virus de l'encéphalomyélite équine du Venezuela (EEV), le virus de la vaccine, *Coxiella burnetii* ou le virus de la fièvre jaune. Un certain nombre de technologies à base d'immunologie ont été utilisées au RDDC Suffield pour faire l'analyse des échantillons de valeur inconnue, l'une d'entre elles étant le dosage immunoenzymatique (ELISA). Des ELISA à capture d'antigènes ont été mis au point pour tous les 10 agents biologiques et ont été utilisés pour faire l'analyse préliminaire des échantillons et un panel d'agents hétérologues a été inclu comme contrôle pour en identifier la spécificité. Cinq agents biologiques de valeur inconnue ont été identifiés par ELISA dont trois au niveau de l'espèce : *B. melitensis, F. tularensis,* and *Y. pestis* et deux au niveau du genre : *Bacillus spp.* Un échantillon contenant *V. cholerae* a produit une réaction faussement négative. Une comparaison des résultats ELISA avec l'identité des organismes des échantillons de valeur inconnue de SIBCA, telle que révélée par le Polygone d'essais de Dugway après les exercices, indiquait que trois des échantillons ont été identifiés correctement, que deux échantillons ont été partiellement identifiés et qu'un échantillon a été identifié incorrectement comme faux négatif.

Executive summary

Introduction: NATO/Partner for Peace (PfP) Forces may be required to support battlefield or peacekeeping operations in areas of the world where biological weapons have been used or where there is a threat of their use. Under such circumstances, samples known to, or suspected of, containing biological agents, are likely to be referred to NATO/PfP laboratories for identification or confirmation of biological agent content. To evaluate the capabilities of NATO/PfP laboratories in identifying biological agents in samples, the NATO Panel VII Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) have sponsored a number of international training exercises in which participant laboratories have been asked to identify agents in sample unknowns.

In January 2002, SIBCA conducted the fourth international training exercise on identification of biological agents. Fourteen NATO/PfP national laboratories participated: Austria, Bulgaria, Canada, Denmark, France, Germany (two laboratories), Italy, the Netherlands, Norway, Poland, Sweden, the United Kingdom, and the United States. The participating laboratory for Canada was Defence R&D Canada – Suffield (DRDC Suffield). DRDC Suffield used a number of different technologies to screen the SIBCA samples, one of which was the enzyme-linked immunosorbant assay (ELISA). This report describes the results obtained in screening SIBCA samples for 10 different biological agents by ELISA.

Results: Antigen-capture ELISAs for *Bacillus anthracis, Coxiella burnetii, Yersinia pestis, Francisella tularensis, Vibrio cholerae, Brucella melitensis,* VEE virus, *Burkholderia mallei,* Vaccinia virus, and Yellow fever virus were used to screen SIBCA samples for homologous agents. Concurrently, respective assays were evaluated for specificity against a panel of heterologous agents.

Five biological agent unknowns were identified by ELISA, three at the species level: *B. melitensis, F. tularensis,* and *Y. pestis,* and two at the genus level: *Bacillus spp.* One sample containing *V. cholerae* produced a false negative reaction.

Significance of results: The results of this report demonstrate that the ELISA is a useful tool for identification of biological unknowns. Results indicated that, at the present time, *Bacillus spp.* cannot be distinguished from each other by immunological methods using the antibodies currently in the DRDC Suffield inventory. Participation of DRDC Suffield in NATO/PfP SIBCA training exercises is valuable, as it provides a means for DRDC Suffield to measure and evaluate, against international standards, in-house capabilities in the identification of biological agents from sample unknowns.

Future goals: Most of the agent ELISAs used in this exercise had not been optimized for sensitivity or specificity, nor had they been evaluated for reactivity with common battlefield materials, or assessed for assay reproducibility. While inclusion of the heterologous agent panel in the SIBCA exercise provided confidence in the specificity of the reagents used in respective agent ELISAs, further work is required to screen each agent assay against additional related and unrelated agents as well as potential assay interferents, and to perform evaluations of assay reproducibility on a statistical basis. In the longer term, each agent

ELISA should be challenged with live agent, to confirm assay sensitivity with live materials. In addition, development of antibodies that could discriminate between *B. anthracis* and other closely related *Bacillus* species would be desirable, as would the investigation of alternative, more sensitive immunological techniques for identification of biological agents in sample unknowns. In addition, the development of multiplexed immunological assay systems that can be used to screen samples simultaneously for a large number of different agents would be beneficial.

Sommaire

Introduction: les Forces du Partenariat pour la Paix de l'OTAN peuvent être appelées à soutenir des opérations de maintien de la paix ou de champs de bataille dans des zones où les armes biologiques ont été utilisées ou bien où il existe une menace qu'elles le soient. Dans ce contexte, les échantillons de valeur connue ou suspectés de contenir des agents biologiques sont en général envoyés à des laboratoires du Partenariat pour la paix de l'OTAN pour que l'agent biologique soit identifié ou confirmé. Pour évaluer les capacités des laboratoires du Partenariat pour la paix de l'OTAN à identifier les agents dans les échantillons, le sous-groupe du Panel VII de NATO d'échantillonnage et l'identification des agents biologiques et chimiques (SIBCA), a parrainé un certain nombre d'exercices internationaux de formation durant lesquels les laboratoires participants ont été requis d'identifier des agents dans des échantillons de valeur inconnue.

En janvier 2002, SIBCA a conduit le quatrième exercice international de formation sur l'identification des agents biologiques. Quatorze laboratoires du Partenariat pour la paix de l'OTAN ont participé : l'Autriche, la Bulgarie, le Canada, le Danemark, la France, l'Allemagne (deux laboratoires), l'Italie, les Pays-Bas, la Norvège, la Pologne, la Suède, la Grande-Bretagne et les États-Unis. Le laboratoire désigné pour le Canada était celui de R & D pour la défense, Canada – Suffield (RDDC Suffield). Le RDDC Suffield a utilisé un certain nombre de technologies pour effectuer les analyses préliminaires des échantillons SIBCA, l'une d'entre elles étant le dosage immunoenzymatique (ELISA) Cet article décrit les résultats obtenus de l'analyse préliminaire des échantillons SIBCA pour 10 agents biologiques différents, par la technique ELISA.

Résultats: On a utilisé les techniques ELISA à capture d'antigènes pour le *Bacillus anthracis*, *Coxiella burnetii*, *Yersinia pestis*, *Francisella tularensis*, *Vibrio cholerae*, *Brucella melitensis*, le virus EEV, *Burkholderia mallei*, le virus de la vaccine et le virus de la fièvre jaune pour effectuer l'analyse préliminaire des échantillons SIBCA pour les agents homologues. On a simultanément évalué des biotests respectifs contre un panel d'agents hétérologues.

On a identifié cinq agents biologiques de valeur inconnue par la technique ELISA, trois au niveau de l'espèce : *B. melitensis, F. tularensis et Y. pestis* et deux au niveau du genre : *Bacillus spp.* Un agent contenant le *V. cholerae* a produit une fausse réaction.

La portée des résultats : Les résultats de cet article indiquent que la technique ELISA est un outil utile pour identifier les inconnus biologiques. Les résultats indiquent qu'à présent, il n'est pas possible de distinguer les *Bacillus spp* entre eux par des méthodes immunologiques qui utilisent actuellement les anticorps de

l'inventaire de RDDC Suffield. La participation de RDDC Suffield aux exercices de formation SIBCA des Partenaires pour la paix de NATO est précieuse ; elle permet au RDDC Suffield de mesurer et d'évaluer les capacités internes de l'identification d'agents biologiques d'échantillons inconnus, par rapport aux normes internationales.

Les objectifs futurs : La plupart des ELISA utilisées dans cet exercice n'ont pas été optimisées pour la sensitivité ou la spécificité et elles n'ont pas non plus été évaluées pour la réactivité avec les matériaux usuels de champs de guerre, ni évaluées pour la reproductibilité des biotests. Alors que l'inclusion de l'exercice du panel d'agents hétérologues dans l'exercice SIBCA a permis de spécifier avec certitude les réactifs utilisés dans les ELISA respectives, de plus amples travaux sont requis pour effectuer l'analyse préliminaire de chaque biotest d'agent contre des agents qui y sont ou n'y sont pas liés ainsi que l'analyse d'interférants potentiels des biotests. De plus amples travaux sont aussi requis pour effectuer des évaluations de la reproductibilité des biotests sur une base de statistique. À plus long terme, chaque technique ELISA devrait être évaluée avec un agent vivant pour confirmer la sensitivité du biotest aux matériaux vivants. De plus, la mise au point d'anticorps qui pourraient faire la discrimination entre les *B. anthracis* et d'autres espèces *Bacillus* qui lui sont proches serait désirable, tout comme l'étude de techniques de solutions de rechange plus sensibles pour l'identification d'agents biologiques dans des échantillons inconnus. La mise au point de systèmes de biotest immunologiques multiplexés qui peuvent être utilisés pour effectuer simultanément l'analyse préliminaire des échantillons d'un grand nombre d'agents différents serait aussi bénéfique.

Table of contents

List of figures

List of tables

Acknowledgements

Introduction

NATO/Partner for Peace (PfP) forces may be required to carry out military or peacekeeping operations in areas of the world where there is a threat of attack with biological agents or where the occurrence of biological attack is suspected or confirmed. Under such circumstances, NATO/PfP forces would be expected to take samples of materials suspected of containing biological agents and to forward same to respective national laboratories, where procedures would be carried out to identify the agent unknowns and to confirm their presence in samples. In order to assess national capabilities in the NATO/PfP laboratories for identification of biological agents in samples, the NATO Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) organized international training exercises in which participating nations were requested to identify, within a given time period, agents in sample unknowns.

The first SIBCA training exercise for biological agents i.e., SIBCA I, was hosted by Dugway Proving Ground (DPG), UT, in March 1999. Samples, consisitng of PBS spiked with biological agents, were number coded by DPG, then shipped to participant nations for analysis. Participant nations were advised that biological agents could consist of any one of the following 10 gamma-irradiated organisms: *Bacillus anthracis, Yersinia pestis, Vibrio cholerae*, Venezuelan Equine Encephalitis (VEE) virus, *Francisella tularensis, Brucella melitensis, Burkholderia mallei*, yellow fever virus, vaccinia virus, or *Coxiella burnetii*. The participating laboratory for Canada was Defence Research Establishment Suffield (DRES)[1]. DRES screened sample unknowns by two different antibody-based identification technologies, the Threshold™ device, a light addressable potentiometric sensor (LAPS), and immunochromatographic assays [1, 2]. In addition, a limited analysis by genetic techniques was also used [3].

A second SIBCA training exercise (SIBCA II), again hosted by DPG, was held in February 2000. Six sample unknowns from the list of 10 agents used in the SIBCA I exercise, again suspended in PBS, were sent to the participating laboratories. Two of the samples also contained common battlefield interferents, either burnt vegetation residue or burnt diesel fuel residue. Two technologies were employed by DRES to assess the samples, one genetic-based method [4] and one antibody-based method, enzyme-linked immunosorbent assay (ELISA) [5].

In February 2001, DPG again hosted a SIBCA training exercise (SIBCA III) in which seven samples, six containing agent and one blank, from the same list of 10 inactivated agents as were used in the previous two SIBCA exercises, were sent to each participating laboratory. Three of the samples were agent suspended in soil, while the remainder were in a PBS matrix. DRDC Suffield employed three different technologies for this exercise, including one genetic-based technique [6] and two antibody-based techniques, namely ELISA [7] and Threshold™ immunoassay [8].

---

[1] DRES: renamed Defence Research and Development Canada – Suffield (DRDC Suffield), in January, 2001

In January 2002, SIBCA conducted the fourth international training exercise on identification of biological agents. Fourteen NATO/PfP national laboratories participated: Austria, Bulgaria, Canada, Denmark, France, Germany (two laboratories), Italy, the Netherlands, Norway, Poland, Sweden, the United Kingdom, and the United States. The designated laboratory for Canada was DRDC Suffield. For the fourth SIBCA exercise (SIBCA IV) (also hosted by DPG), six swab samples were sent to participating countries. Each swab was sealed inside a 15 mL polypropylene tube and all tubes were held inside a sealable plastic bag. Due to pressing commitments, the samples, which were received in January, 2002, could not be analyzed upon receipt. In September, 2002, analyses of the samples were initiated by both genetic and immunological methods. This report describes the results obtained on screening of SIBCA IV sample unknowns by ELISA, complemented by limited Threshold^{TM} assays and bacterial stain techniques.

Antigen-capture ELISAs were used to analyze SIBCA IV sample unknowns for all 10 SIBCA agents. Assays for each agent were configured in such a way that liquid sample unknowns were screened in parallel with homologous agents (positive controls) and a panel of heterologous agents, thus providing concurrent confirmation of specificity of respective agent assays. Threshold^{TM} assays were performed on SIBCA sample 126 in an effort to confirm the ELISA results for this sample that differed from those obtained by genetic methods. A Gram stain and a malachite green spore stain were also performed in efforts to ascertain the identity of SIBCA sample 126. Five biological agent unknowns were identified by ELISA in SIBCA samples, three at the species level and two at the genus level. A comparison of the ELISA results with the identity of organisms in SIBCA sample unknowns, as revealed by US DPG following the exercise, indicated that three organisms, *B. melitensis*, *F. tularensis*, and *Y. pestis*, had been correctly identified in samples 160, 201, and 219, respectively. In addition, a *Bacillus spp.* was correctly identified in samples 126 and 161. One false negative, *V. cholerae* (sample 128) was also observed.

Materials and methods

SIBCA test samples

Pre-exercise information

Six SIBCA sample unknowns adsorbed onto cotton swabs (numbered 126, 128, 160, 161, 201, and 219) were received at DRDC Suffield from US DPG on 30 Jan 02. Participants were informed that samples would contain any one of the following killed (cobalt-irradiated) agents: *B. anthracis, C. burnetii, Y. pestis, F. tularensis, V. cholerae, B. melitensis,* VEE virus, *B. mallei*, vaccinia virus, or yellow fever virus. Ten working days, not necessarily consecutive, were allowed for completion of the analyses, after which time, results were to be forwarded to DPG for collation.

Sample preparation

Each of the six swab samples was received at DRDC Suffield sealed inside a 15 mL snap-cap polypropylene tube and enclosed in a zippered plastic bag. Due to other pressing commitments, the samples were not analyzed immediately. Rather, the plastic bags containing the tubes were wrapped in aluminum foil and stored at 4°C until 16 Sep 02 when the samples were reconstituted and analysis begun.

Samples were reconstituted as follows: 0.5mL PBS, pH 7.4, (Invitrogen Canada Inc., Burlington, ON) was added to each tube containing a swab and the samples were vortexed 12 times for 10-second bursts. The tubes were then centrifuged at 3000 rpm for one minute (Beckman GS-SR centrifuge, GH-3.8 rotor). The liquid was visually inspected and findings recorded. Both the swabs and liquid were then transferred to sterile 1.5 mL microfuge tubes and the swabs dipped and squeezed five times against the sides of the microfuge tubes. The swabs were then removed from the microfuge tubes and placed back into their original tubes, which were then sealed, wrapped in aluminum foil, and stored at 4°C. The remaining liquid was aliquoted into sterile microfuge tubes in 100 uL (or less) volumes, wrapped in foil, and stored at 4°C until analysis commenced.

Post-exercise identity of sample unknowns

After all laboratories had completed their analyses and the results had been reported, participants were informed by DPG of the identities of agents present in SIBCA sample unknowns. The identities of the agents in the six sample unknowns received by Canada are presented in Table 1.

Table 1. Agents in SIBCA exercise IV samples[2]

SAMPLE NO.	AGENT
126	*Bacillus thuringiensis, israeliensis*
128	*Vibrio cholerae*
160	*Brucella melitensis*
161	*Bacillus anthracis*
201	*Francisella tularensis*
219	*Yersinia pestis*

Enzyme-linked immunosorbant assays

Antibodies

Unlabelled antibodies

The following antibodies were purchased from commercial sources: *V. cholerae*, polyvalent rabbit antiserum (Inaba, Ogawa), catalogue no. M-2432-50B, lot no. 142625 (Lee Laboratories, Grayson, GA); *V. cholerae* 01, mouse monoclonal IgG, catalogue no. VCM-5261-5, lot no. VC6N11, 0.25 mg/mL (Austral Biologicals, San Ramon, CA); *C. burnetii*, positive control human serum, catalogue no. 4030-02-01, lot no. CBP018 (Integrated Diagnostics, Baltimore, MD); and yellow fever virus, mouse monoclonal ascites (clone 2D12), catalogue no. MAB984, lot no. 20060494 (Chemicon International, Temicula, CA).

Antibody stocks developed under DRDC Suffield contract by SciLab Consulting Inc., Redcliff, AB [9] were as follows: *B. anthracis*, goat IgG, lot no. SC97Ant001, 5 mg/mL, serial no.[3] CABAC70P0090797; *B. anthracis*, rabbit IgG, lot no. SC97Ant002, 5 mg/mL, serial no. CABAC71P0090797; *Y. pestis*, goat IgG, lot no. SC97YP001, 4 mg/mL, serial no. CAYER3810/08/99; *Y. pestis,* rabbit IgG, lot no. SC97YP002, 3 mg/mL, serial no. CAYER9310/08/99; *B. melitensis*, goat IgG, lot no. SC95BM001, 15 mg/mL, serial no. CABru7925/08/9908:52:06; *B. melitensis*, rabbit IgG, lot no. SC97Mel002, 10 mg/mL, serial no. CABru11P0090797; *B. mallei*

---

[2] Information provided by US DPG following reporting to DPG of SIBCA IV results
[3] Serial no. assigned by DRDC Suffield MOU database

mallein, goat IgG, lot no. SC97M1001, 10 mg/mL, serial no.
1CaBur21P0090797; *B. mallei* mallein, rabbit IgG, lot no. SC97M1002, 10
mg/mL, serial no. CABur16P0090797; VEE virus, goat IgG, lot no. SC
97VEE002, 4 mg/mL, serial no. CaVen84P0090797; VEE virus, rabbit IgG,
10 mg/mL, lot no. SCVEE001, serial no. CaVen6P0000529; vaccinia virus,
goat IgG, lot no. SC97V001, 5 mg/mL, serial no. CAVac61P0090797; and
vaccinia virus, rabbit IgG, lot no. SC97002, 5 mg/mL, serial no.
CaVac69P0090797.

The following antibodies were produced under DRDC Suffield contract by
Canadian Bioconcepts Inc., Saanichton, BC [10]: *F. tularensis,* bovine IgG,
6.7 mg/mL, serial no. CAFRA5716/08/99; and *F. tularensis,* rabbit IgG, 2.6
mg/mL, serial no. CaFra60P014079.

C. burnetii rabbit IgG, lot no. 030899-01, 5.9 mg/mL and yellow fever rabbit
IgG, lot no. 040199-01, 5.0 mg/mL were gifts from the Naval Medical
Research Center (NMRC), Bethesda, MD.

Labelled antibodies

The following horseradish peroxidase-labelled antibodies were purchased
from Sigma Aldrich Canada Ltd., Oakville ON: rabbit anti-human IgG (whole
molecule, lot no. 068H4897); goat anti-rabbit IgG (whole molecule, lot no.
40H8822); and rabbit anti-goat IgG (whole molecule, lot no. 90H8990).
Horseradish peroxidase-labelled goat anti-mouse IgG + IgM (H and L chain),
lot no. 17011200 was purchased from Caltag Laboratories, Burlingame, CA.

Antibody purification

V. cholerae rabbit antiserum (Lee Laboratories), *C. burnetii* human positive
control serum (Integrated Diagnostics), and yellow fever virus mouse ascites
(Chemicon International) were purified by a Pierce Nab^{TM} protein G spin
chromatography kit (Biolynx Inc., Brockville, ON). Procedures followed
were those described in the manufacturer's protocol. Antibodies were
purified in 100 µL batches and samples were eluted with 400 µL of elution
buffer, after which, samples were immediately neutralized by the addition of
sodium phosphate, pH 8.0. Fractions containing maximum protein content,
typically fraction one, were collected for use in ELISA. Protein
concentrations of IgGs resulting from the above described purifications were:
V. cholerae rabbit IgG: 4.6 mg/mL; *C. burnetii* human IgG: 2.0 mg/mL; and
yellow fever virus mouse monoclonal IgG: 2.7 mg/mL.

Antibodies produced by Scilab Consulting Inc. were purified on a Bio-Gel^{R}
Protein G Fast Flow Gel column (Bio-Rad Laboratories, Mississauga, ON) by
a High-Performance Liquid Chromatography system (Spectral Physics, San
Jose, CA) [9]. Antibodies produced by Canadian Bioconcepts Inc. were

purified on a BioRad rProtein A™ column by a Gilson Low Pressure Liquid
Chromatography system [10].

Antigens

The following antigens were purchased from commercial sources: Mutacol Berna[R] *V. cholerae* live oral vaccine, strain CVD 103-HgR, lot no. 01532401 (Berna Products Corp., Mississauga, ON): stock *V. cholerae* antigen (~2-10x10^9 cfu/mL) was prepared by re-suspending a single vaccine dose in 100 mL Dulbecco A PBS (Oxoid Inc., Nepean, ON); yellow fever virus strain 17D, live attenuated vaccine, lot no. C0229AA (Connaught Laboratories, Willowdale, ON): stock yellow fever virus antigen was prepared by re-suspending a 5 dose vial in 3.0 mL of 0.9% sodium chloride (diluent provided); and *C. burnetii*, Phase 1 antigen, catalogue no. 534P1-V (Vero), lot no. CBPh1-030900WX, formalin inactivated, 1mg/mL (PanBio InDx Inc., Baltimore, MD).

The following cobalt-irradiated antigen stocks were gifts from DPG: *Y. pestis*, India 195/P strain (F1+), 3.6×10^8 cfu/mL; *F. tularensis*, Schu 4 strain, lot no. 95306, 7.8×10^8 cfu/mL; *B. anthracis*, Vollum strain, lot no. 96092, 1.3×10^8 cfu/mL; vaccinia virus, Lister strain, 1×10^7 pfu/mL; and *B. melitensis*, type 2, 4.3×10^9 cfu/mL.

B. mallei mallein complement fixation antigen (ophthalmic), serial no. 91-94, exp date 95.12.31, was a gift from Animal Diseases Research Institute, Nepean, ON. VEE strain TC83, originally obtained from the US Army Medical Institute of Infectious Diseases, was grown and purified at DRDC Suffield. DRES Run 1, pool 2 (105 µg/mL) was used as antigen stock for the ELISA.

Format

ELISAs for the identification of all 10 SIBCA agents were developed in indirect (antigen capture) assay format. By this method, capture antibody (CAb) is adsorbed to the solid phase and is used to capture the target antigen or sample unknown. Agent ELISAs were used to screen the SIBCA trial samples for homologous agents and, concurrently, to evaluate respective assays for specificity against a panel of heterologous agents.

Procedures

Agent ELISAs performed on the SIBCA samples included positive and negative (no antigen) controls, the sample unknowns, and a standard panel of heterologous agents comprising all the possible agents listed by US DPG for inclusion in the exercise. To provide sufficient volume for ELISA analysis, the SIBCA samples were diluted 1:42 in PBS prior to use. Samples were assayed one agent per plate, two agents per day. All samples were tested in replicates of three wells. To minimize day-to-day

variability, heterologous agents and positive control antigens were prepared in advance at working concentrations and stored at −70 °C until used. Antigen that was unused on a given day was discarded.

ELISAs were performed in 96-well NUNC™ Maxisorb microtiter plates purchased from Canadian Life Technologies, Burlington, ON. Positive control antigens and sample unknowns were detected with unlabelled detector antibody (DAb) and indicated by enzyme-labelled indicator antibody (IAb). Washes were performed using a Bio-Tek ELX-50 autostrip washer (Fisher Scientific, Edmonton, AB). Wash steps consisted of five cycles of washing with a volume of 300 µL wash buffer (PBS containing 0.1% bovine serum albumin (Roche Diagnostics, Laval, QC) and 0.1% Tween-20) per wash cycle. Antigen, or sample unknown, DAb, and IAb were diluted, as required, in ELISA buffer (PBS containing 2% BSA and 0.1% Tween-20). Unless otherwise specified, all incubation steps were at 37 °C for 1 hr.

Wells were coated with 100 µL of CAb in coating buffer (carbonate-bicarbonate buffer, 0.05M, pH 9.6) and incubated at 4° C overnight. Plates were washed, then blocked by the addition of 300 µL of blocking buffer (PBS containing 2% BSA), and incubated. Plates were washed, then 100 µL of antigen, or sample unknown, was added, and the plates incubated. Plates were again washed, 100 µL of DAb was added, plates were incubated, then washed. One hundred µL of IAb was added and the plates were incubated, then washed. One hundred µL of substrate solution ((2.2'-azino-di- (3-ethyl-benzthiazoline sulfonate) (ABTS) (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD)) or 3,3',5,5'tetramethylbenzidine ((TMB) (Kirkegaard and Perry Laboratories Inc.)) was added and the plates incubated at room temperature for 30 min, after which the coloured reaction product was measured in an automated plate reader (see below).

Immunoreagents

The identity and working concentrations of CAbs, DAbs, IAbs, and positive control antigens used in respective agent ELISAs, are shown in Table 2. Assays were performed in duplicate with one plate receiving as substrate, ABTS and the other TMB. The IAb concentration for use with ABTS was as used previously for SIBCA III [7]. The IAb concentration for use with TMB was 1/5000, with the exception of the *Y. pestis* assay in which TMB had been previously optimized at 1/3000.

Table 2. Working concentrations of capture, detector, and indicator antibodies, and positive control antigens used in agent ELISAs

AGENT ELISA	CAB	DAB	IAB (ABTS SUBSTRATE)	IAB (TMB SUBSTRATE)	POSITIVE CONTROL ANTIGEN
B. anthracis	goat α-B.anthracis 10 µg/mL	rabbit α-B.anthracis 15 µg/mL	goat α-rbt-HRP 1:1000	goat α-rbt-HRP 1:5000	B.anthracis, vollum: 1.3x10^7 cfu/mL, 6.5x10^6 cfu/mL, 3.25x10^6 cfu/mL
Y. pestis	goat α-Y.pestis 10 µg/mL	rabbit α-Y.pestis 25 µg/mL	goat α-rbt-HRP 1:1000	goat α-rbt-HRP 1:3000	Y.pestis-India (F1+): 3.6x10^6 cfu.mL, 1.8x10^6 cfu.mL, 0.9x10^6 cfu.mL
B. melitensis	goat α-B.melitensis 15 µg/mL	rabbit α-B.melitensis 20 µg/mL	goat α-rbt-HRP 1:1000	goat α-rbt-HRP 1:5000	B.melitensis, type 2: 4.3x10^7 cfu/mL, 2.15x10^7 cfu/mL, 1.1x10^7 cfu/mL
F. tularensis	bovine α-F.tularensis 15 µg/mL	rabbit α-F.tularensis 20 µg/mL	goat α-rbt-HRP 1:1000	goat α-rbt-HRP 1:5000	F.tularensis, Shu 4: 7.8x10^7 cfu/mL, 3.9x10^7 cfu/mL, 1.95x10^7 cfu/mL
V. cholerae	rabbit α-V.cholerae 15 µg/mL	mouse α-V.cholerae 15 µg/mL	goat α-ms-HRP 1:3000	goat α-ms-HRP 1:5000	V.cholerae vaccine 2-10x10^7 cfu/mL, 1-5x10^7 cfu/mL, 0.5-2.5x10^7 cfu/mL
B. mallei	goat α-B.mallei mallein CF Ag 15 µg/mL	rabbit α-B.mallei mallein CF Ag 20 µg/mL	goat α-rbt-HRP 1:1000	goat α-rbt-HRP 1:5000	B. mallei: mallein CF Ag 1:100, 1:200, 1:400

VEE	goat α-VEE 10 µg/mL	rabbit α-VEE 20 µg/mL	goat α-rbt-HRP 1:1000	goat α-rbt-HRP 1:5000	VEE DRES Run 1, pool 2: 12 µg/mL, 6 µg/mL, 3 µg/mL
Vaccinia	goat α-vaccinia 15 µg/mL	rabbit α-vaccinia 15 µg/mL	goat α-rbt-HRP 1:1000	goat α-rbt-HRP @ 1:5000	Vaccinia, Lister: 0.5×10^7 pfu/mL, 0.25×10^7 pfu/mL, 0.125×10^7 pfu/mL
C. burnetii	rabbit α-*C.burnetii* 15 ug/mL	human α-*C. burnetii* 15 µg/mL	rbt α-hu-HRP 1:5000	rbt α-hu-HRP 1:5000	*C. burnetii*, Ph 1 50 µg/mL, 25 µg/mL, 12.5 µg/mL
Yellow fever	mouse α-yellow fever 15 ug/mL	rabbit α-yellow fever 20 µg/mL	goat α-rbt-HRP 1:1000	goat α-rbt-HRP 1:5000	Yellow fever vaccine: 1:2, 1:4, 1:8

Heterologous agent panel

Agent ELISAs were evaluated for specificity by testing for cross-reactivity with a standard panel of heterologous antigens encompassing all antigens included in the SIBCA exercise (Table 3).

Agent concentrations for use in the heterologous agent panel were determined by titrating each agent in the respective agent ELISA and selecting a concentration that was at least one log above background absorbance + two standard deviations (for organisms) or 10 times the end-point concentration (for proteins and dilutions) (Fulton R. E., unpublished data). Titrations were performed using both ABTS and TMB, respectively, as substrates.

Table 3. Panel of heterologous agents used to evaluate specificity of agent ELISAs

AGENT	CONCENTRATION
B. anthracis, vollum	1.0×10^6 cfu/mL
Y. pestis, India (F1+)	1.0×10^6 cfu/mL
B. melitensis, type 2	5.0×10^6 cfu/mL
F. tularensis, Schu 4	1.0×10^6 cfu/mL
V. cholerae, vaccine	$2.0\text{-}10.0 \times 10^6$ cfu/mL4
B. mallei mallein, CF Ag	1:1000
VEE TC83, vaccine (DRES Run 1, pool 2)	5.0 µg/mL
Vaccinia, Lister	1.0×10^6 pfu/mL
C. bumetii, Ph 1	5 µg/mL
Yellow fever, vaccine	1:1000

Data Acquisition, reduction, and analysis

ELISA reaction products were read at A_{405} nm (ABTS) or A_{650} nm (TMB) in a Molecular Devices Thermomax^R automated plate reader (Fisher Scientific). Preliminary data analysis was performed using Softmax 3.0 software (Molecular Devices, Menlo Park, CA). Data was then exported to Microsoft Excel 7.0 for reduction and statistical analysis. Reduced data was further exported to Grapher 3 (Golden Software Inc., Golden, CO) for plotting of graphs.

Statistics

Samples were tested in replicates of three wells. Percent coefficients of variation (CVs) among replicates were calculated and found to average around 2% with the single highest CV being 5.6%. Unless otherwise indicated, data points represent the mean of three determinations.

---

[4] Dilution error: should have been $2.0\text{-}10.0 \times 10^7$ cfu/mL; $2.0\text{-}10.0 \times 10^6$ cfu/mL previously found too low for ELISA detection by homologous antibody (unpublished data); insufficient reagent to repeat assay.

ELISA readings were considered positive if the mean absorbance reading was significantly greater than the background absorbance reading. Statistical significance was established at 20% above the background (no antigen control) absorbance. This reading represents a value of ten times the average CV among replicates. Thus, a signal was considered positive if the value of the sample signal to background signal was 1.2 or greater.

Threshold™ assays

Materials

Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) and Triton X-100 were from BDH Chemicals (Toronto, ON). Bovine serum albumin (BSA), sodium chloride (NaCl), sodium hydroxide (NaOH), Tween-20, and urea were from Sigma Chemical Co. (St. Louis, MO). Streptavidin (Scripps Laboratories, San Diego, CA), 10 mg/mL in distilled water, was prepared previously (for SIBCA I in 1999) and stored at 4°C. Biotinylated Threshold ™ sticks, N-hydroxysuccinimide ester of dinitrophenyl biotin, and N-hydroxysuccinimide ester of carboxyfluorescein were from Molecular Devices Corp. (Menlo Park, CA).

Antigens and antibodies

B. anthracis, vollum, inactivated antigen was as described above. *Bacillus subtilis var niger (B. globigii)*, lyophilized, was from DPG; spores were reconstituted in PBS (1 mg/mL) and diluted as necessary.

Rabbit anti-*B. anthracis* (SciLab Consulting Inc.) (as described for ELISA assays) was conjugated previously (Apr 02) with fluorescein and biotin, in accordance with procedures provided in the Threshold™ operators' manual, and subsequently stored at 4°C. Concentrations of fluorescein and biotin in the conjugated products were 0.304 mg/mL and 0.178 mg/mL, respectively. Similarly, rabbit anti-*B. globigii* (DRDC Suffield) was previously conjugated (May 95) with fluorescein and biotin and subsequently stored at 4 °C; concentrations of fluorescein and biotin in these conjugated products were 0.575 mg/mL and 0.362 mg/mL, respectively. Lyophilized anti-fluorescein urease-conjugated antibody was purchased from Molecular Devices Corp. Prior to use, urease-conjugated antibody was reconstituted in 30 mL assay buffer to a stock concentration of 7.5 µg/mL.

Reagents

Wash buffer consisted of 10 mM sodium phosphate (pH 6.5), 150 mM NaCl, and 0.05% Tween-20. Assay buffer consisted of 10 mM sodium phosphate (pH 7.0), 150 mM NaCl, 0.025% Triton X-100, and 0.1% BSA. Wash and assay buffers were stored at 4°C, no longer than one week.

The substrate solution was 100 mM urea in wash buffer (pH 6.5), prepared fresh daily.

Reagents were used at the following concentrations: biotin-labeled antibody (100 ng/test site), fluorescein-labeled antibody (100 ng/test site), and streptavidin (500 ng/test site).

Equipment

The assay apparatus was a commercially available LAP sensor marketed under the name Threshold™ Unit (Molecular Devices Corp.). The instrument is capable of simultaneously processing four membrane sticks consisting of eight reaction test sites per stick. The instrument is controlled by a Sidus P133 microcomputer and custom software supplied by Molecular Devices Corp.

Analysis

The reaction scheme of the Threshold™ assay was as previously described [1].

Threshold analysis of the SIBCA samples was limited to sample 126. Prior to analysis of SIBCA sample 126, both the *B. globigii* and the *B. anthracis* antibody sets were tested for possible cross-reactivity with both *B. anthracis* and *B. globigii* positive control antigens at concentrations of 200 ng/test site. In order to minimize the effect of stick-to-stick variation, assays for each bacterium were confined to a single stick. Each antigen assay was performed in replicates of four, including four negative assay buffer controls. Volumes of 400 µL of each antigen (positive control) and assay buffer (negative control) were pipetted into separate tubes. A volume of 400 µL of reagent mixture was added to each tube, the contents vortexed, and the mixtures allowed to incubate at room temperature for 30 minutes. During the incubation step, streptavidin was diluted to 600 ng/mL with assay buffer and the anti-fluorescein urease conjugate was diluted 1:10 from the stock. At the completion of the 30 minute incubation step, 4 mL of the streptavidin solution was added to each tube and 1 mL of the reaction mixture was pipetted into appropriate locations on the membrane filtration unit, and filtered under low vacuum. After filtration, wash buffer (2 mL) was added to each well and filtered under high vacuum to completion. The vacuum was set to "low" and anti-fluorescein urease conjugate (1 mL) was added to each test site. The reagent was removed by filtration and each test site was again washed with wash buffer (2 mL) under high vacuum. The membrane sticks were removed from the filtration compartment, then inserted into the reader compartment containing the LAP sensor and the substrate solution. The rate of pH change with respect to time at the surface of the sensor was monitored as the rate of change of the surface potential with respect to time in µV/sec.

Prior to assay, SIBCA sample 126 was diluted 1:420 in assay buffer and then tested for the presence of either or both *B. anthracis* and *B. globigii* (three replicates of SIBCA sample 126, three negative controls, two positive controls). The assay method for SIBCA sample 126 was similar to the assays for *B. globigii* and *B. anthracis* positive controls except the volumes used for SIBCA sample 126 were adjusted for the number of replicates.

Statistics

Samples were tested in replicates of two or three wells, as described above. Standard deviations were only calculated for samples tested in replicates of three. Threshold™ readings were considered positive if the ratio of sample signal to negative control signal was 1.2 or greater.

Stains

Gram stain

A Gram stain kit (Becton Dickinson Microbiology System, Cockeysville, MD) was used to stain SIBCA sample 126. Included on the slide provided in the kit were Gram positive and Gram negative controls consisting of *Staphylococcus aureaus* and *Escherichia coli*, respectively. A loopful of SIBCA sample 126 was spread across several numbered sections of the microscope slide and allowed to air dry for 5 minutes. The sample was heat-fixed to the slide and the slide cooled to room temperature. The slide was flooded with gentian violet (0.4%), the latter being allowed to remain on the slide for 1 minute. The gentian violet was removed under running water and the slide then flooded with iodine solution. After 1 minute, the slide was rinsed with water and held at an angle while decolourizer (1:1 ethanol:acetone) was applied until the solvent flow became colourless. The slide was rinsed with water and flooded with safranin solution (0.25% safranin in 20% ethanol), which was allowed to remain on the slide for one minute. The slide was again rinsed with water and blotted dry. The slide was viewed under a light microscope (Zeiss Instruments Ltd., Calgary, AB) and examined for the presence of Gram positive and/or Gram negative bacteria.

Spore stain

A small loopful of SIBCA sample 126 was spread across a microscope slide and allowed to air dry for 5 minutes. Similarly, a loopful of a suspension of *B. globigii* (1 mg/mL in PBS) (DPG) was spread across a separate slide and air-dried, to serve as a positive control. The samples were heat-fixed and the slides cooled to room temperature. The slides were placed over a beaker of rapidly boiling water and flooded with malachite green (5%). Slides were left over the boiling water for 6 minutes, after which time the excess stain was rinsed off with water. The slides were then flooded with safranin and, after 30 seconds, rinsed with water. The slides were viewed under a light microscope (Nikon Eclipse E400, Calgary, AB) and photos taken of the magnified spores.

Results

SIBCA samples

All reconstituted SIBCA samples, on visible inspection, were observed to be clear with the exception of sample 126, which was cloudy with particulates suspended and adherent to the inner wall of the tube.

Enzyme-linked immunosorbant assays

Results obtained by challenge of 10 agent ELISAs with the six SIBCA sample unknowns, the heterologous agent panel, and homologous agent positive controls are summarized in Table 4 and graphically represented in Figures 1-10. Since the majority of the agent assays had not been optimized for either ABTS or TMB substrates, the decision as to whether to report the results of the ABTS or TMB substrate assays was based on which assay exhibited the higher signal to background ratio in the low positive control antigen. The differences between low positive control antigen signal to background ratios for assays using TMB versus ABTS differed by a low of 8% to a high 17% with the average difference being 13%. In addition, The VEE assay produced saturated absorbances in all wells when TMB was employed (unpublished data). Hence, for *F. tularensis*, *B. mallei*, and VEE, the ABTS results were reported, whereas, the TMB results were reported for the remaining agents.

Positive controls for all 10 agent ELISAs (*B. anthracis*, *C. burnetii*, *B. melitensis*, *F. tularensis*, *Y. pestis*, *B. mallei*, vaccinia, VEE, yellow fever, and *V. cholerae*) produced positive reactions in respective homologous assays, thus confirming the validity of each of the agent assays for identification of respective homologous agents. In addition, all 10 agent ELISAs were shown not to cross-react with any of the heterologous agents in the panel at the concentrations tested (Table 3) (Figs. 1-10)

Four SIBCA samples were positive for a single agent in four of the agent ELISAs. Hence, sample 160 was positive in the *B. melitensis* assay, sample 161 was positive in the *B. anthracis* assay, sample 201 was positive in the assay for *F. tularensis*, and sample 219 was positive for *Y. pestis*. In addition, sample 126 was positive for three agents, *B. anthracis*, *B. mallei*, and *V. cholerae*. However the signal to background ratio in sample 126 for *B. anthracis* was approximately 10 times higher than it was for either of the other two antigens, *B. mallei* or *V. cholerae*. No other positive reaction was observed with any SIBCA sample in any of the agent ELISAs.

The observation that the ELISA reactivity in the *B. anthracis* assay was very strong and that this sample contained particulate material on visual inspection, combined with the negative result obtained for *B. anthracis* by genetic analysis, led to the suspicion that sample 126 might contain a *Bacillus spp.* that was cross-reactive with the *B. anthracis* ELISA. To investigate this theory, sample 126 was submitted to the follow-on studies by Threshold™ assay and histochemical analysis.

Table 4. ELISA results: SIBCA samples

AGENT ELISA	POSITIVE CONTROL	SIBCA SAMPLE NO.						CROSS-REACTIVITY WITH HETEROLOGOUS AGENTS
		126	128	160	161	201	219	
B. anthracis	+	+	-	-	+	-	-	none
Y. pestis	+	-	-	-	-	-	+	none
B. melitensis	+	-	-	+	-	-	-	none
F. tularensis	+	-	-	-	-	+	-	none
V. cholerae	+	+	-	-	-	-	-	none
B. mallei	+	+	-	-	-	-	-	none
VEE	+	-	-	-	-	-	-	none
Vaccinia	+	-	-	-	-	-	-	none
C. bumetii	+	-	-	-	-	-	-	none
Yellow fever	+	-	-	-	-	-	-	none

Threshold™ assays

The results for the Threshold™ assays of SIBCA sample 126 are shown in Table 5. Sample 126 was positive by Threshold™ assay for both B. anthracis and B. globigii. To determine whether the Threshold™ assays for B. anthracis and B. globigii cross-reacted, the B. anthracis assay was tested with B. globigii antigen and the B. globigii assay was tested with B. anthracis antigen. The results are shown in Table 6. The assay for B. anthracis cross-reacted with B. globigii antigen at the concentration examined. Similarly, the assay for B. globigii cross-reacted with B. anthracis antigen.

Table 5. Threshold™ assay results: SIBCA sample 126

THRESHOLD™ ASSAY	SAMPLE	SIGNAL/NEG. CONTROL	RESULT
B. anthracis	126	2.5	+
	B. anthracis (pos. control)	39.8	+
B. globigii	126	2.1	+
	B. globigiiI (pos. control)	12.1	+

Table 6. Evaluation of cross-reactivity between B. anthracis and B. globigii assays by Threshold™

THRESHOLD™ ASSAY	SAMPLE	SIGNAL/NEG. CONTROL	CROSS-REACTIVITY
B. anthracis	B. globigii	1.5	yes
	B. anthracis (pos. control)	20.0	
B. globigii	B. anthracis	3.1	yes
	B. globigii (pos. control)	13.5	

Bacterial stains

The Gram stain of sample 126 showed that Gram positive bacteria (stained purple) were present in this sample. The Gram stain of the positive and negative controls included in the kit showed purple cocci and red rods, respectively. The malachite green spore stain of sample 126 showed the presence of spores (stained green) (Figure 11). The malachite green stain of the positive control (*B. globigii*) showed spores (stained green) and cellular debris (stained pink) (Figure 12). The size of the spores in sample 126 appeared, at the same microscope magnification, to be larger in size than the *B. globigii* spores.

Agents identified in sample unknowns

Table 7 is a summary of the agents identified by ELISA in the SIBCA IV samples, compared with the agent content of samples as revealed by US DPG following the SIBCA exercise.

In three of the six samples (sample nos. 160, 201, and 219), the agents present were correctly identified by ELISA. In two other samples (sample 126 and 161), a *Bacillus spp.* organism was correctly identified by ELISA, Threshold™ assay, and bacterial staining methods. Two false positive reactions were also noted by ELISA, both in sample 126, but these were relatively weak signals and did not interfere with the correct identification of a *Bacillus spp.* in this sample. One false negative identification was made by ELISA, in sample 128.

Table 7. Agents identified compared with known agent content of SIBCA IV samples

SIBCA SAMPLE NO.	AGENT PRESENT[5]	AGENT IDENTIFIED
126	*B. thuringiensis, israeliensis*	*Bacillus* spp.
128	*V. cholerae*	-
160	*B. melitensis*	*B. melitensis*
161	*B. anthracis*	*Bacillus* spp.
201	*F. tularensis*	*F. tularensis*
219	*Y. pestis*	*Y. pestis*

---

[5] Information provided by US DPG following SIBCA exercise

Discussion

Each of the agent ELISAs used in SIBCA IV was challenged, not only with positive control antigen, but also with a panel of heterologous antigens comprising all of the agents included in the SIBCA exercise. The purpose for inclusion of the heterologous agent panel was to control for assay specificity, since most of the ELISAs used in this exercise had not undergone extensive optimization and characterization. By this method, it was found that none of the agent antibodies used in this exercise cross-reacted with any of the heterologous agents at the concentrations tested. Concentrations of agents for use in the heterologous agent panel were determined prior to the SIBCA exercise by titrating each antigen for detection sensitivity by ELISA against the respective homologous antibodies. In order that the concentration of heterologous agents not be in excess, which would encourage the occurrence of non-specific reactions, concentrations were selected in the agent titration curve that were approximately 10 times the "cut-off" absorbance (background + two standard deviations). By this means, non-specific reactions in the heterologous agent panel, as were observed in previous SIBCA exercises [5, 7], were averted. It should be noted, however, that due to an inadvertent error in dilution, the concentration of *V. cholerae* in the heterologous panel was 10 times less than the intended value of $2\text{-}10\times10^7$ cfu/mL.

Well-to-well reproducibility in this exercise was much better than seen in previous SIBCA exercises [2, 5, 7], where variability sometimes reached greater than 15%, necessitating the removal of aberrant data prior to analysis. Well-to-well variability among replicates in SIBCA IV ranged from 0.2% to 5.6% with an average CV of 2%. The problem of extreme variability encountered in previous years is believed to be traceable to the size of the repeater pipette cartridge. By reducing the size of the cartridge from 5.0 mL capacity, used in previous SIBCA exercises, to 1.25 mL capacity, used in SIBCA IV, ELISA CVs were reduced to 2%. Furthermore, multiple operators routinely achieved CVs of approximately 2.0-3.0% when using the 1.25 mL capacity cartridges. These findings rule out sources of error previously proposed [7] including irregularities in plate surface, pipetting errors, and operator inconsistencies.

The agents present in SIBCA samples 160, 201, and 219 were correctly identified by ELISA as *B. melitensis, F. tularensis,* and *Y. pestis,* respectively. These results were consistent with those obtained by genetic analysis [11], thus allowing Canada to report identification of agents in these samples at the NATO Confirmed[6] level. However, *V. cholerae,* identified correctly in sample 128 by genetic methods, was not identified in this sample by ELISA. Similarly, the *V. cholerae* ELISA did not identify *V. cholerae* in the heterologous agent panel at a concentration of $2\text{-}10 \times 10^6$ cfu/mL (Figure 10). This would suggest that *V. cholerae* was present in sample 128 at a concentration less than or equal to $2\text{-}10\times10^6$ cfu/mL. The ELISA for *V. cholerae* had not been optimized prior to the SIBCA exercise. However, the assays for both *B. anthracis* and *Y. pestis* had been optimized prior to the exercise and concentrations in the order of 10^3 cfu/mL were readily observed in these assays. While, this does not mean that

---

[6] NATO SIBCA level of identification [28] where Confirmed identification means identification by two different methods in the presence of a positive control.

an optimized *V. cholerae* assay would exhibit the same sensitivity, it still remains a possibility that optimization might enhance the limit of detection of *V. cholerae*

The agent present in sample 161 (*B. anthracis*) was correctly identified by ELISA at the genus level as a *Bacillus spp.* Although this agent produced a positive response in the *B. anthracis* assay, it could not be definitively identified as *B. anthracis* due to the cross-reactivity in this assay with other members of the *Bacillus spp.*, as noted in the following paragraphs. This result was consistent with results obtained by genetic analysis where the agent was identified as *B. anthracis* [11].

The ELISA of sample 126 showed a strong absorbance for *B. anthracis*. Similarly, the Threshold™ assay of this sample tested positive for *B. anthracis*, as well as *B. globigii*. Genetic analysis of this sample, however, showed no traces of *B. anthracis* [11]. Because of the positive immunological response of sample 126 to both *B. anthracis* and *B. globigii* and because previous work at DRDC Suffield had shown cross-reactivity by ELISA among closely-related *Bacillus* strains (Fulton, R.E., unpublished data), it was decided to submit sample 126 to further analysis by performing histochemical studies. Accordingly, Gram and spore stains were performed. The finding of bacteria or spores in sample 126 might account for the immunological observations and would be consistent with the observation that sample 126, on visual inspection, was cloudy and particulate. Results of bacterial staining revealed that sample 126 contained Gram positive, cylinder-shaped bacteria, as well as spores that were slightly larger than *B. globigii* spores (Dan Dragon, personal communication). This finding, coupled with the immunological and genetic results, led to the speculation that the sample contained a *Bacillus spp.* that cross-reacted with the *B. anthracis* antibodies incorporated in the ELISA and Threshold™ assays used in this exercise. Previous studies at DRDC Suffield had shown that the *B. anthracis* ELISA cross-reacted with *B. cereus, B. thuringiensis*, and *B. globigii* antigens (Fulton, R.E., unpublished data). Based on the further observation, albeit subjective, that the spores in sample 126 were larger than *B. globigii* spores, the conclusion was reached that sample 126 contained a *Bacillus* organism that was neither *B. anthracis* nor *B. globigii*. The revelation, post-exercise, that sample 126, in fact, contained *B. thuringiensis* is consistent with the immunological and histochemical findings in this exercise, and with observations made in previous studies that *B. anthracis* and *B. thuringiensis* cross-react immunologically, and that *B. thuringiensis* spores are larger than *B. globigii* spores (Dan Dragon, personal communication).

As noted above, the *B. anthracis* ELISA used in this exercise cross-reacted with *B. cereus, B. thuringiensis*, and *B. globigii* antigens (Fulton, R.E., unpublished data). Immunological cross-reactivity among members of the *Bacillus spp.* has been well documented by others. In fact, differentiation among the *Bacillus spp.* has long posed a dilemma for bacteriologists, and many aspects of the clinical and taxonomic characterization of bacilli still remain unresolved [13, 14]. The two major human pathogenic bacilli are *B. anthracis*, the causative agent of anthrax, and *B. cereus*, a food poisoning organism. *B. anthracis* and *B. cereus* share a high degree of genetic similarity as demonstrated by DNA-DNA hybridization studies [15]. Furthermore, the 16s and 23s rRNA sequences of these two species are almost identical [16, 17]. Other *Bacillus spp.* reported to cross-react with *B. anthracis* and *B. cereus* are *B. thuringiensis*, an insect pathogen used commonly on crops for insect control [18] and *B. globigii* [19]. Using the technique of amplified fragment length polymorphism, Jackson et al.

performed phylogenic analysis of *B. anthracis* and related *Bacillus spp.*, and showed a close genetic relationship (distance) between *B. anthracis*, *B. thuringiensis*, and *B. cereus* [20]. Other investigators have noted that reactivity of *B. globigii* in immunoassay is associated primarily with a soluble component of reconstituted *B. globigii*, rather than the bacterium itself, and have suggested that cross-reactivity among the *Bacillus spp.* might be due to sharing of a common soluble, non-protein antigen e.g. a carbohydrate [12].

The development of species-specific *B. anthracis* immunoassays requires the production of specific reagents i.e., polyclonal or monoclonal antibodies that can distinguish *B. anthracis* from commonly-occurring nearest neighbour species. Although monoclonal antibodies to vegetative cell [23] and spore components [21] have been produced, these reagents were found not to be specific in recognition of *B. anthracis* spores. Although immunoassays form the current foundation for many rapid pathogen technologies, existing antibody reagents for identification of *B. anthracis* spores may be limited in ability to distinguish *B. anthracis* from environmentally ubiquitous near- and distant-neighbour species such as *B. cereus*, *B. subtilis*, *B. thuringiensis*, and *B. globigii* [21, 22]. In a study to evaluate the sensitivity of a suite of existing polyclonal and monoclonal antibodies produced against anthrax spore antigens, it was found that none of the antibodies tested was completely specific in recognition of the anthrax spore surface [19]. Furthermore, attempts to produce specific antibodies by adsorption of *B. anthracis* antibodies with cross-reacting nearest-neighbour *Bacillus spp.* have met with only limited successes [24, 25, 26, 27]. In similar studies conducted at DRDC Suffield it was found that adsorption of *B. anthracis* polyclonal antibodies with cross-reactive *Bacillus spp.* substantially reduced or removed unwanted cross-reactivity but at the expense of loss in titer of *B. anthracis*-specific reactivity (Fulton, R.E., unpublished data). In spite of the universal problems in acquiring immuno-reagents that can specifically identify *B. anthracis* and distinguish it from closely related *Bacillus spp.*, it is this very property of non-exclusivity that allowed us to identify a *Bacillus spp.* organism in SIBCA sample 126, while no organism was identified by the genetic methods used in this exercise. [11]. This is a good example of the value of applying redundant technologies to the task of identification of biological agents in samples. In a report describing the consolidated results obtained by each of the laboratories participating in SIBCA IV, it was revealed that Canada was the only country to correctly identify *Bacillus spp.* in sample 126 [29].

Conclusions

Participation in NATO/PfP SIBCA IV has provided DRDC Suffield with the opportunity to assess current capabilities in the immunological identification of biological agent unknowns. Agent ELISAs, in antigen-capture format, were used to screen SIBCA IV samples for all 10 possible SIBCA agents. In addition, a Threshold™ assay and Gram and spore stains were performed on sample 126. Results indicated that five of the six samples were correctly identified by ELISA; three at the species level and two at the genus level. One additional sample exhibited a false negative response. It is clear from the results of this exercise, that ELISA is a useful tool for identification of biological unknowns and that, in spite of the lack of species-level specificity of the *B. anthracis* ELISA, as currently configured, this assay has utility in identification of organisms at the genus level i.e., *Bacillus spp.*

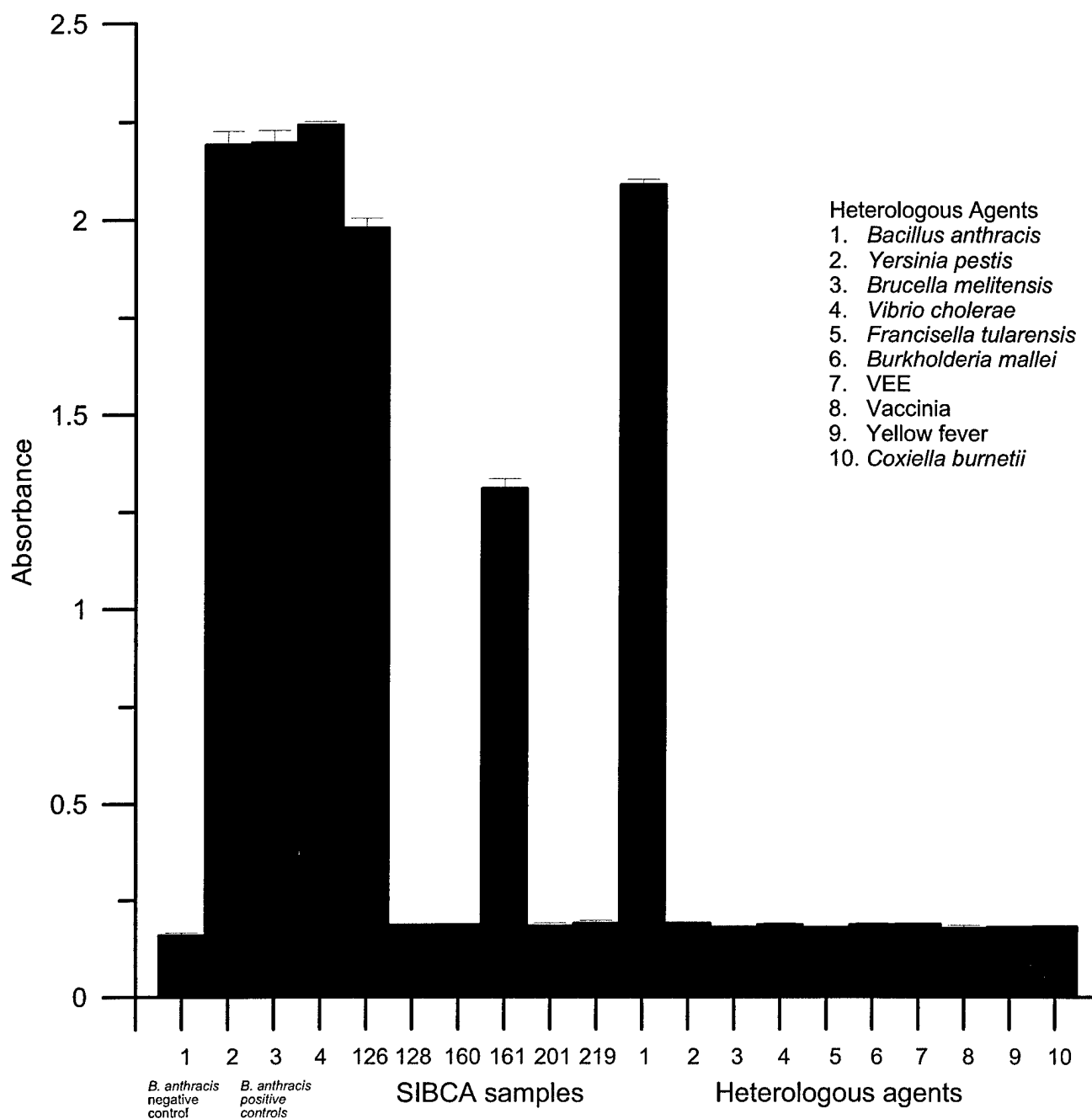This page left intentionally blank.

Figure 1. B. anthracis ELISA challenged with SIBCA IV sample unknowns and the heterologous agent panel (1-10). Positive controls 2, 3, and 4 were $3.25x10^6$, $6.5x10^6$, and $1.3x10^7$ cfu/mL, respectively. Substrate was TMB. Error bars represent the mean +/- one standard deviation.

Heterologous Agents
1. *Bacillus anthracis*
2. *Yersinia pestis*
3. *Brucella melitensis*
4. *Vibrio cholerae*
5. *Francisella tularensis*
6. *Burkholderia mallei*
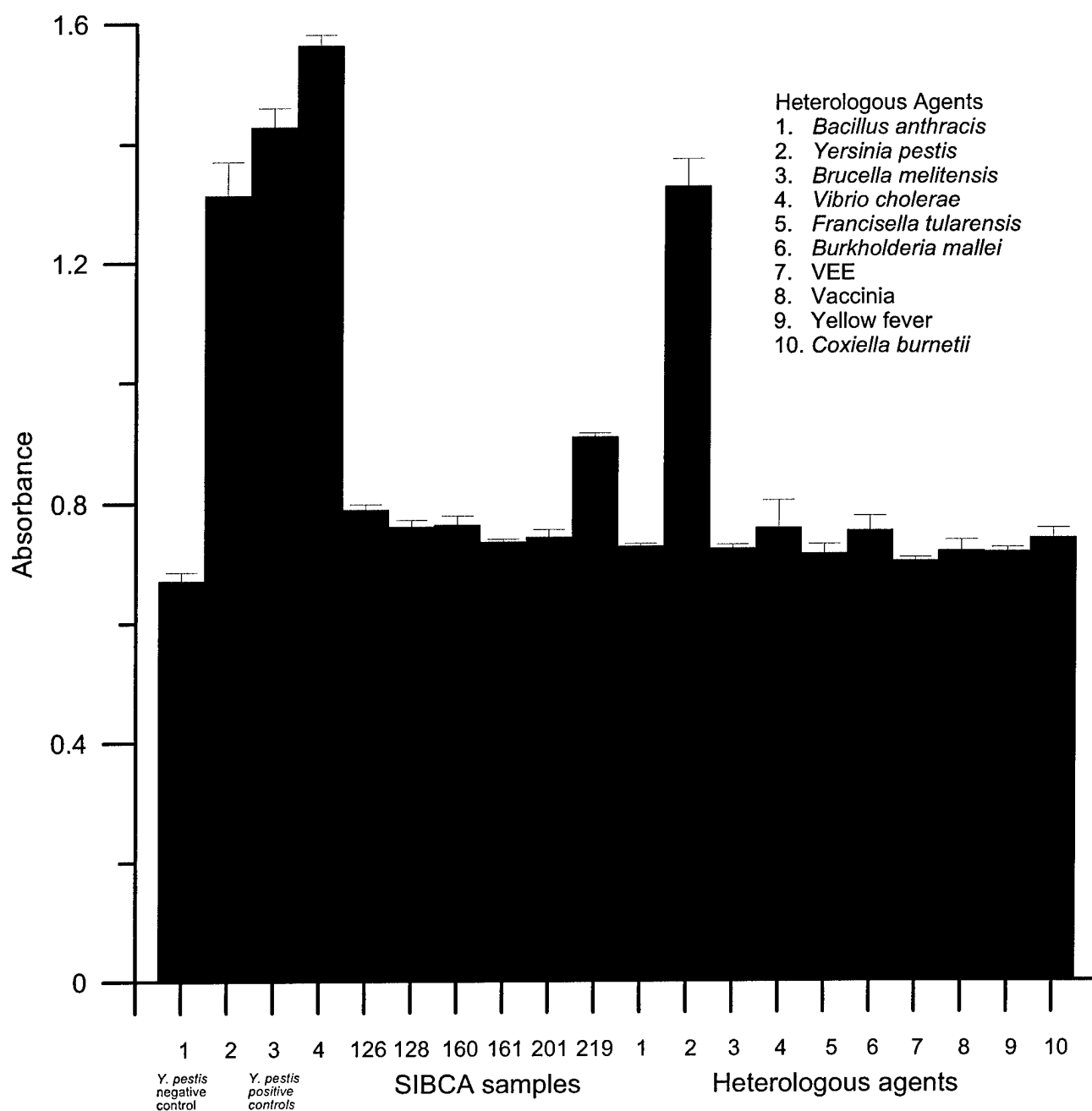7. VEE
8. Vaccinia
9. Yellow fever
10. *Coxiella burnetii*

Figure 2. Y. pestis ELISA challenged with SIBCA IV sample unknowns and the heterologous agent panel (1-10). Positive controls 2, 3, and 4 were 0.9x10^5, 1.8 x10^6, and 3.6x10^6 cfu/mL, respectively. Substrate was TMB. Error bars represent the mean +/- one standard deviation.

Heterologous Agents
1. *Bacillus anthracis*
2. *Yersinia pestis*
3. *Brucella melitensis*
4. *Vibrio cholerae*
5. *Francisella tularensis*
6. *Burkholderia mallei*
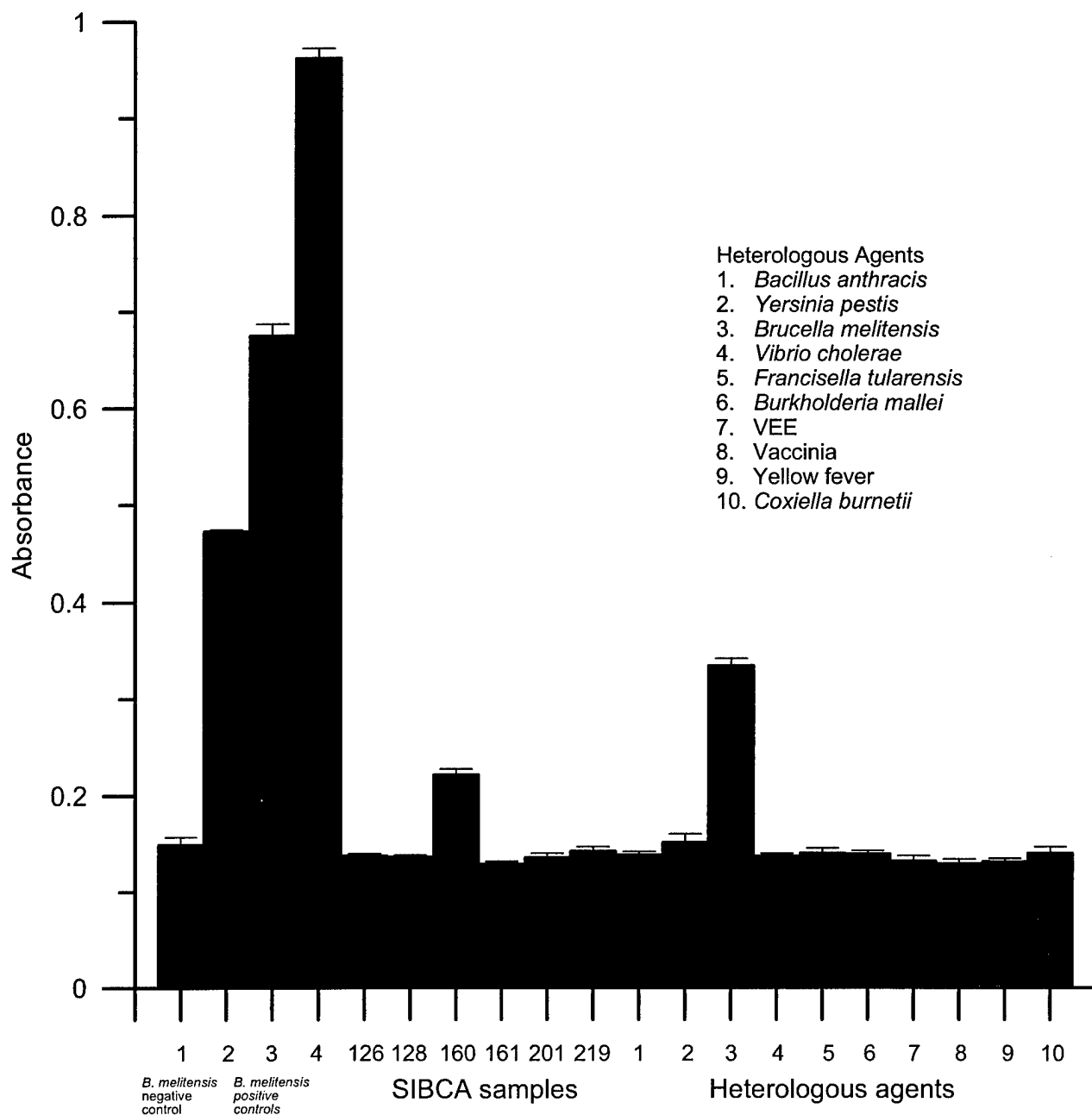7. VEE
8. Vaccinia
9. Yellow fever
10. *Coxiella burnetii*

Figure 3. B. melitensis ELISA challenged with SIBCA IV sample unknowns and the heterologous agent panel (1-10). Positive controls 2, 3, and 4 were 1.1x10^7, 2.15 x10^7, and 4.3x10^7 cfu/mL, respectively. Substrate was TMB. Error bars represent the mean +/- one standard deviation.
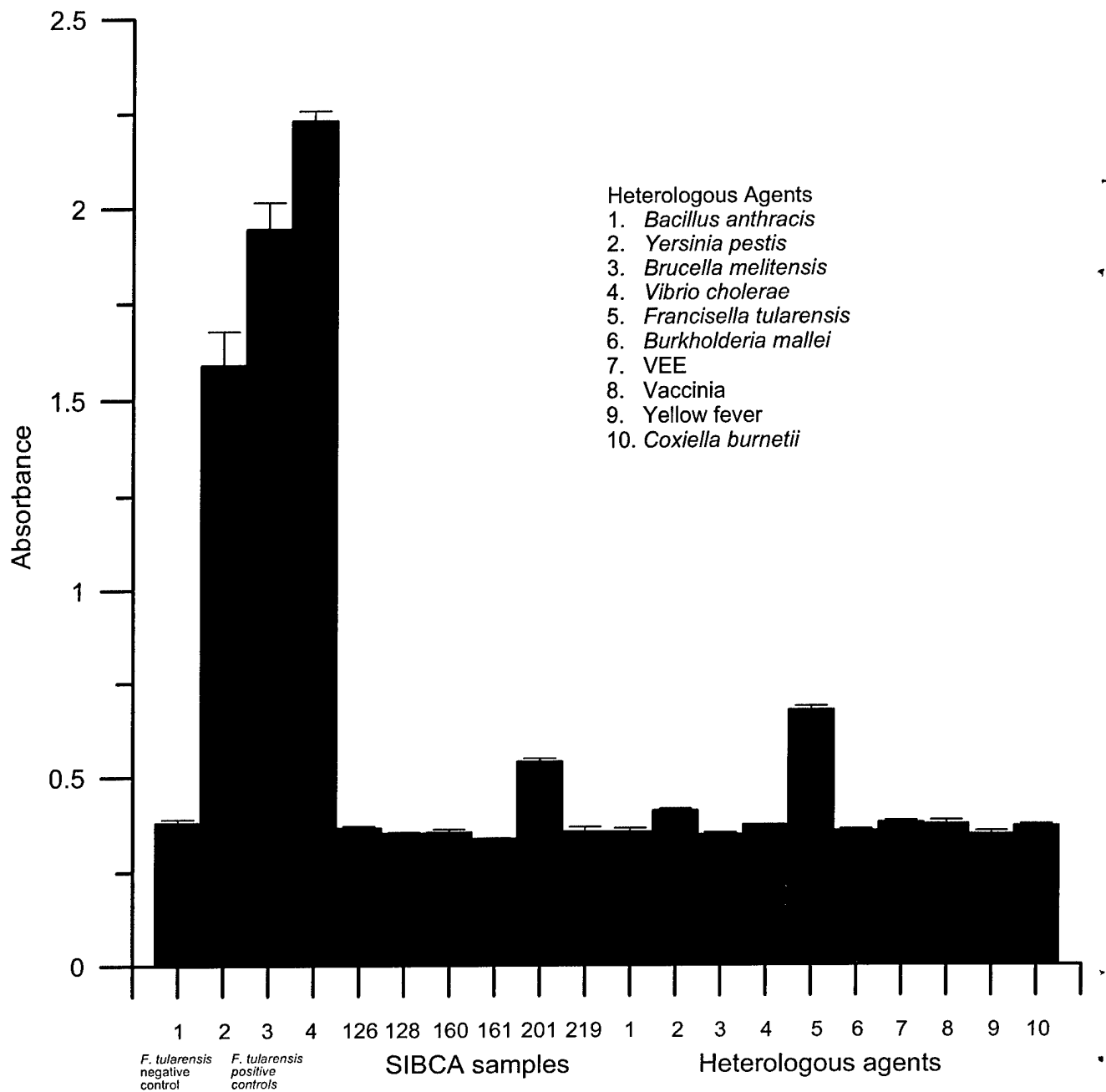
Figure 4. F. tularensis ELISA challenged with SIBCA IV sample unknowns and the heterologous agent panel (1-10). Positive controls 2, 3, and 4 were 1.95×10^7, 3.9×10^7, and 7.8×10^7 cfu/mL, respectively. Substrate was ABTS. Error bars represent the mean +/- one standard deviation.

Heterologous Agents
1. *Bacillus anthracis*
2. *Yersinia pestis*
3. *Brucella melitensis*
4. *Vibrio cholerae*
5. *Francisella tularensis*
6. *Burkholderia mallei*
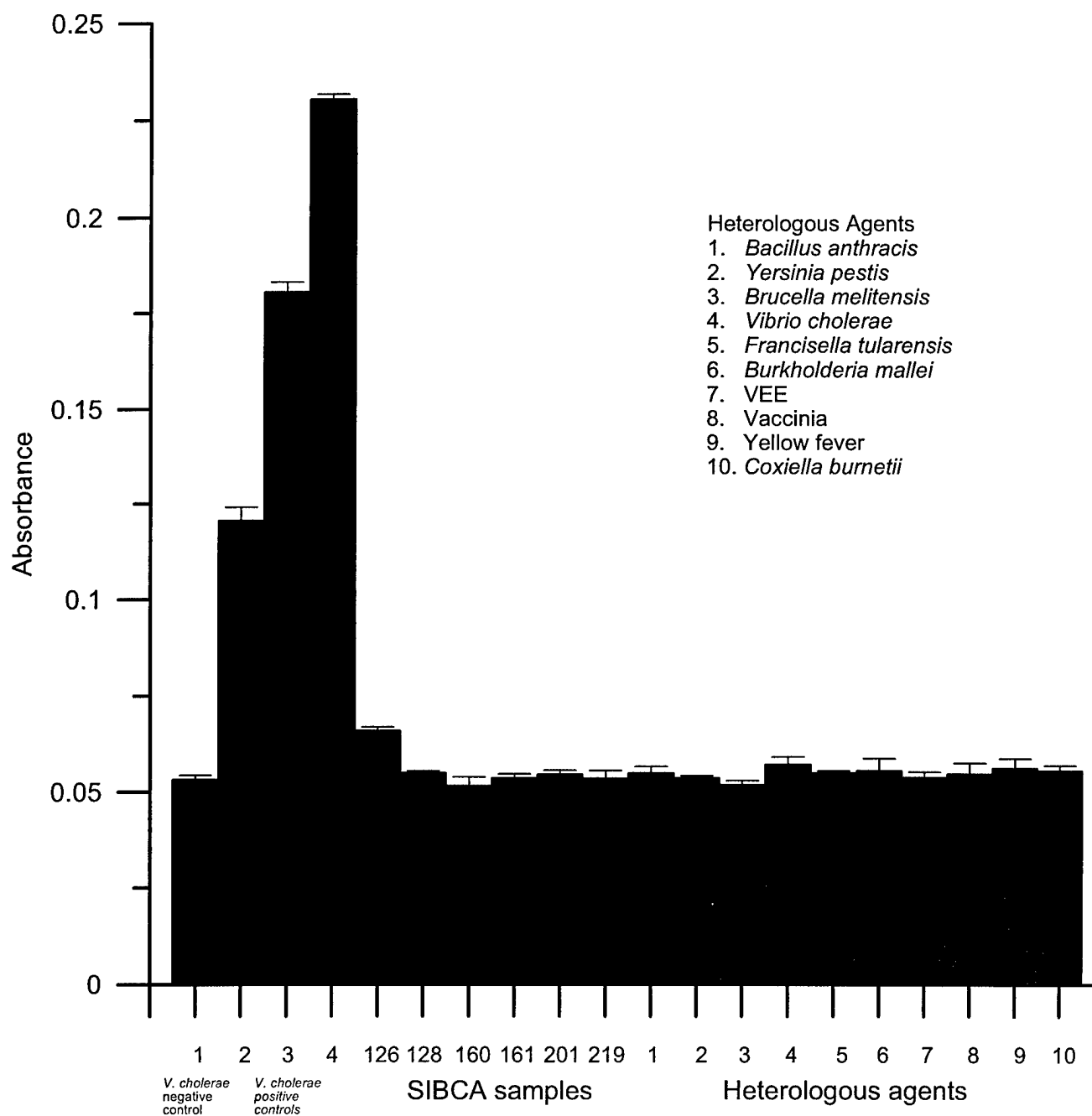7. VEE
8. Vaccinia
9. Yellow fever
10. *Coxiella burnetii*

Figure 5. V. cholerae ELISA challenged with SIBCA IV sample unknowns and the heterologous agent panel (1-10). Positive controls 2, 3, and 4 were 0.5-2.5x10^7, 1-5 x10^7, and 2-10x10^7 cfu/mL, respectively. Substrate was TMB. Error bars represent the mean +/- one standard deviation.
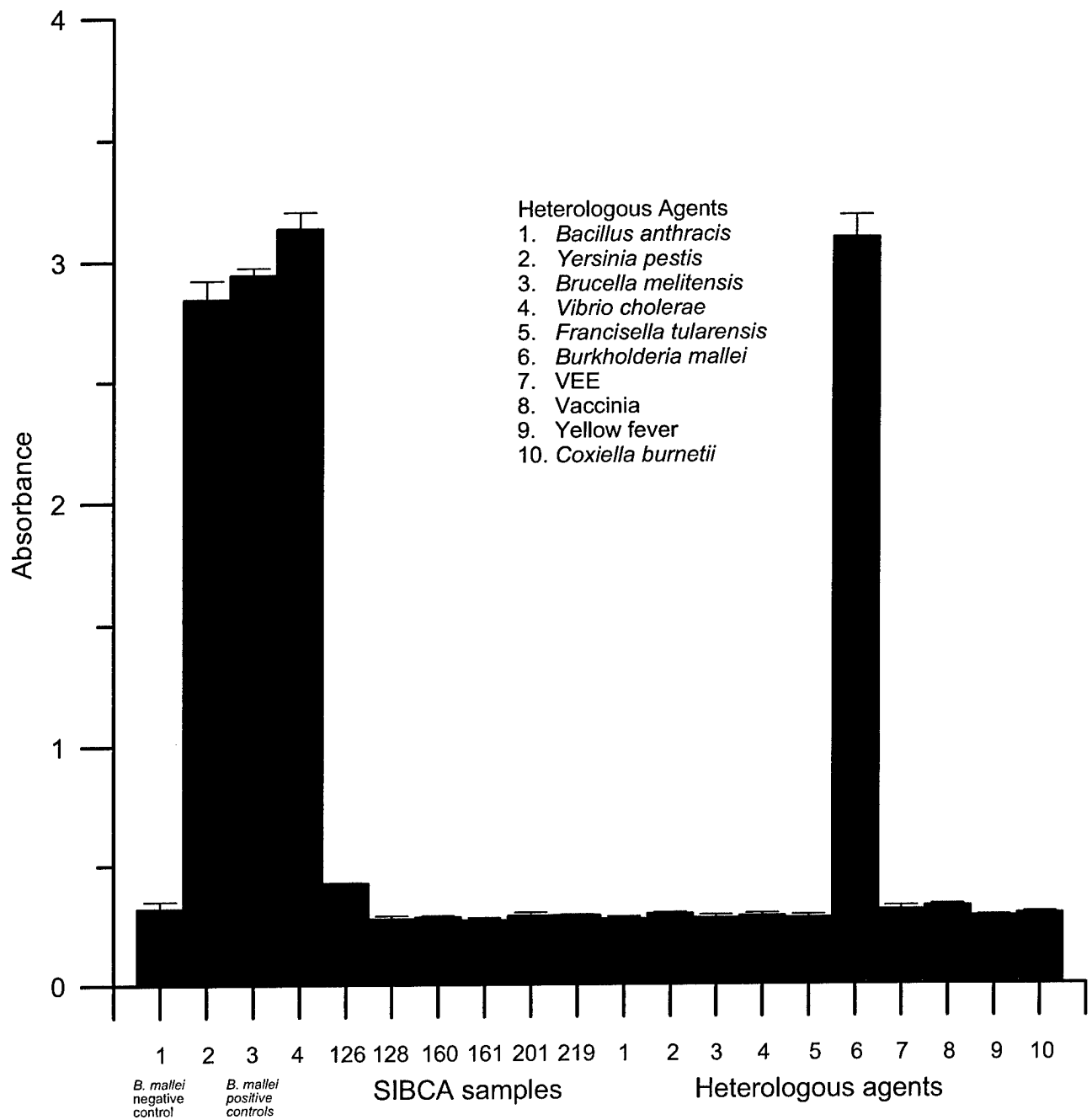
Figure 6. *B. mallei ELISA challenged with SIBCA IV sample unknowns and the heterologous agent panel (1-10). Positive controls 2, 3, and 4 were 1:400, 1:200, and 1:100 dilutions, respectively. Substrate was ABTS. Error bars represent the mean +/- one standard deviation.*
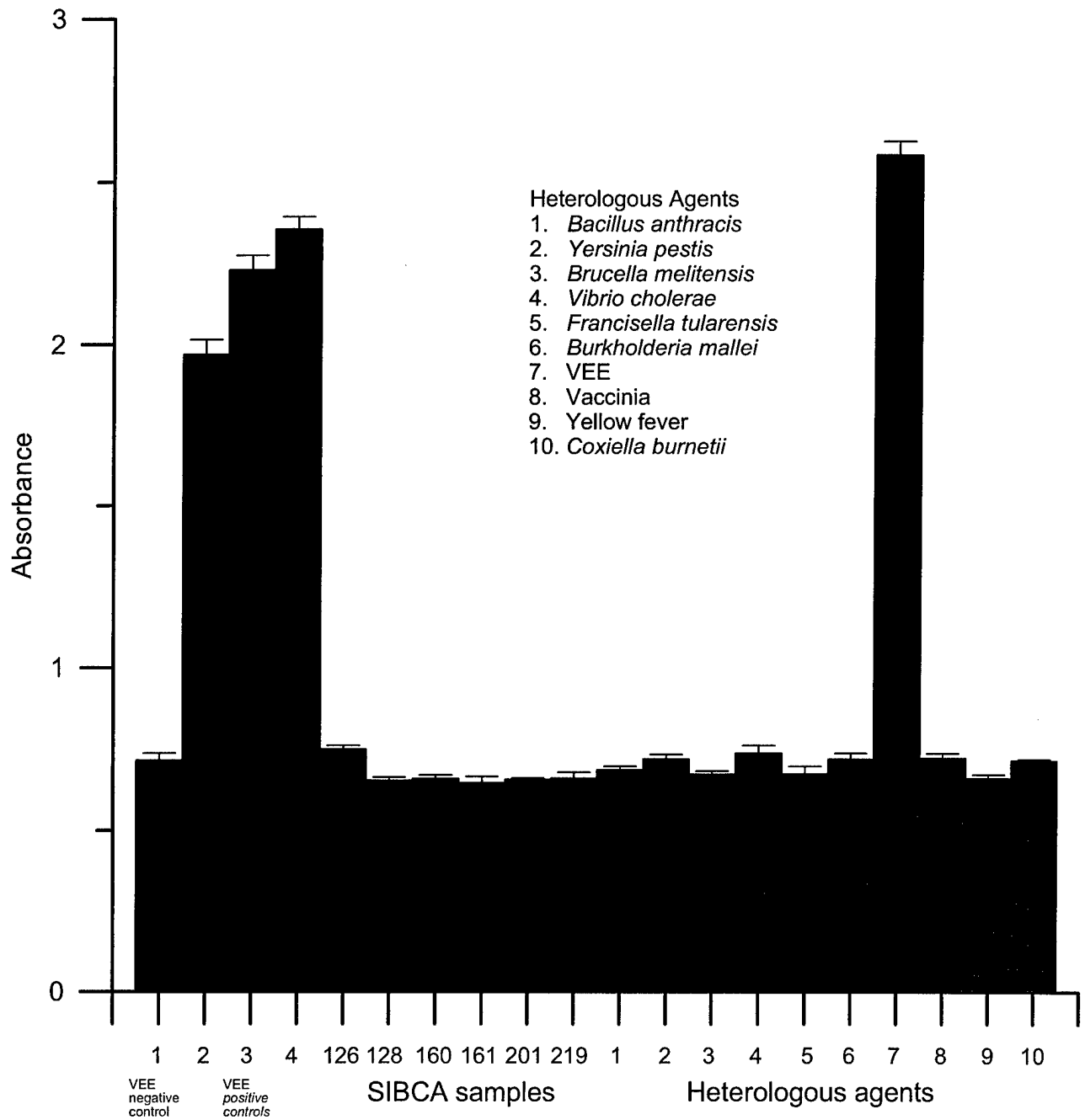
Figure 7. VEE ELISA challenged with SIBCA IV sample unknowns and the heterologous agent panel (1-10). Positive controls 2, 3, and 4 were 3, 6, and 12 ug/mL, respectively. Substrate was ABTS. Error bars represent the mean +/- one standard deviation.
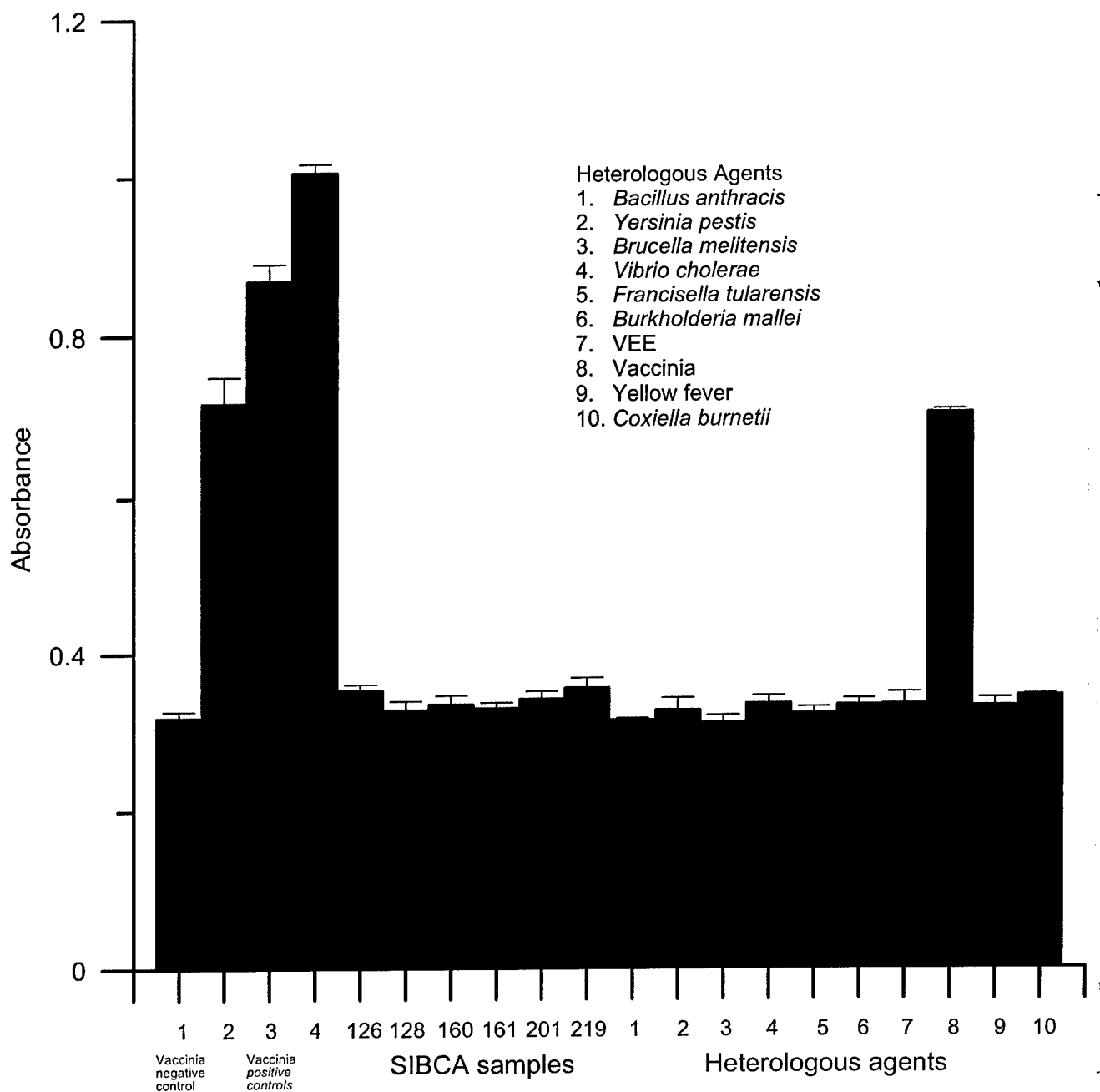
Figure 8. Vaccinia ELISA challenged with SIBCA IV sample unknowns and the heterologous agent panel (1-10). Positive controls 2, 3, and 4 were 0.125x10⁷, 0.25 x10⁷, and 0.5x10⁷ pfu/mL, respectively. Substrate was TMB. Error bars represent the mean +/- one standard deviation.
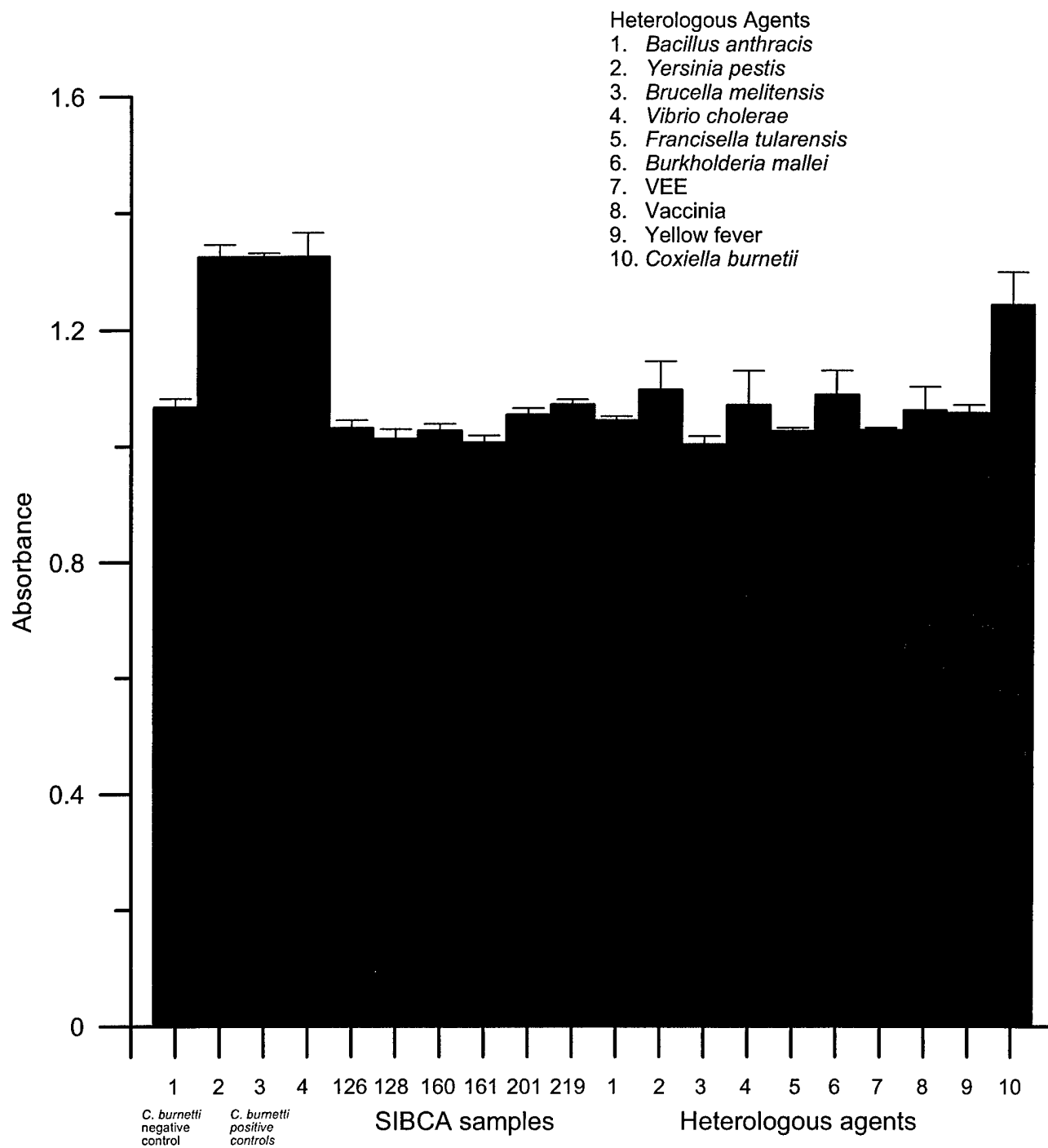
Figure 9. C. burnetii ELISA challenged with SIBCA IV sample unknowns and the heterologous agent panel (1-10). Positive controls 2, 3, and 4 were 12.5, 25, and 50 ug/mL, respectively. Substrate was TMB. Error bars represent the mean +/- one standard deviation.

Heterologous Agents
1. *Bacillus anthracis*
2. *Yersinia pestis*
3. *Brucella melitensis*
4. *Vibrio cholerae*
5. *Francisella tularensis*
6. *Burkholderia mallei*
7. VEE
8. Vaccinia
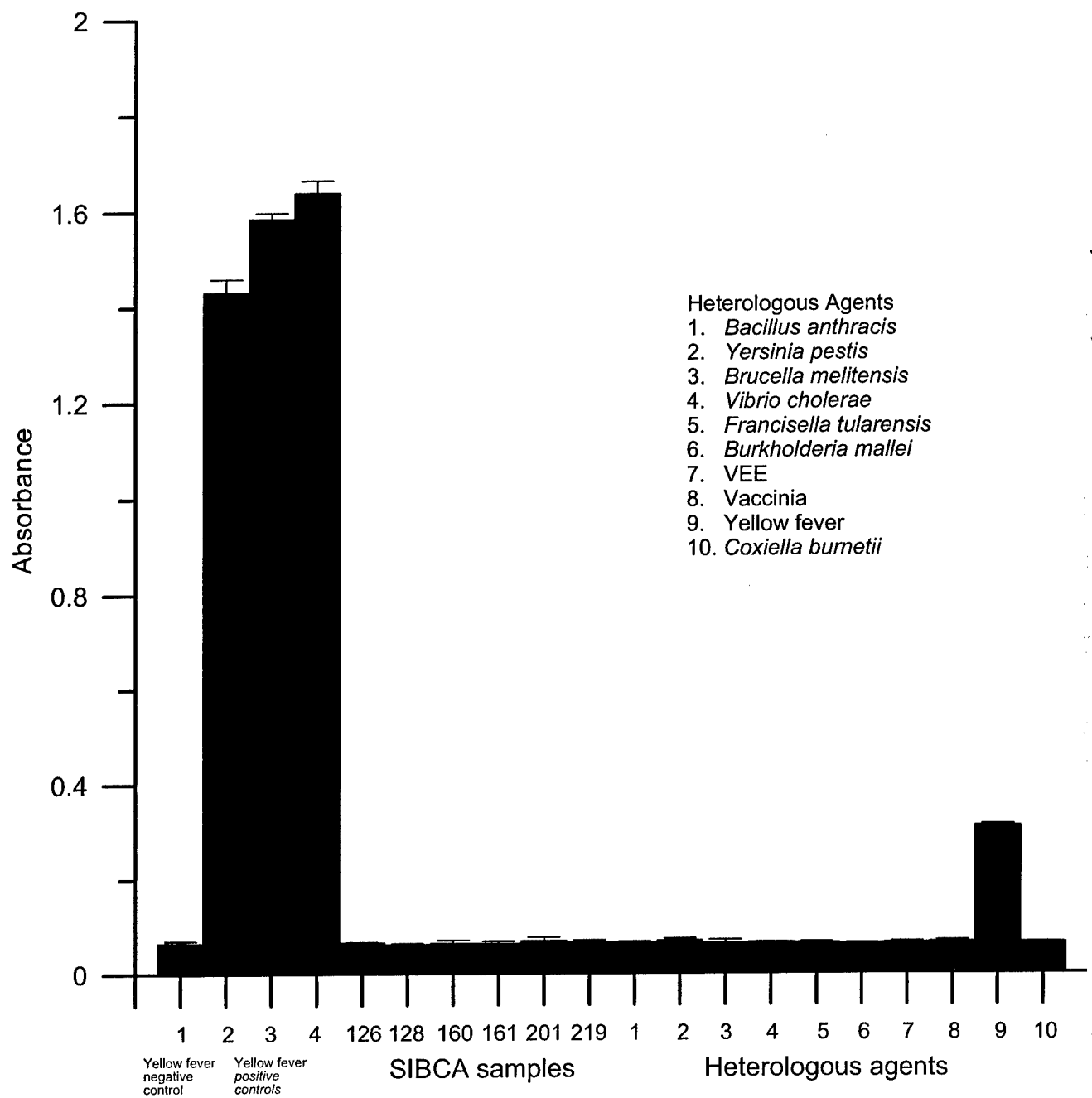9. Yellow fever
10. *Coxiella burnetii*

Figure 10. Yellow fever ELISA challenged with SIBCA IV sample unknowns and the heterologous agent panel (1-10). Positive controls 2 ,3, and 4 were 1:8, 1:4, and 1:2 dilutions, respectively. Substrate was TMB. Error bars represent the mean +/- one standard deviation.
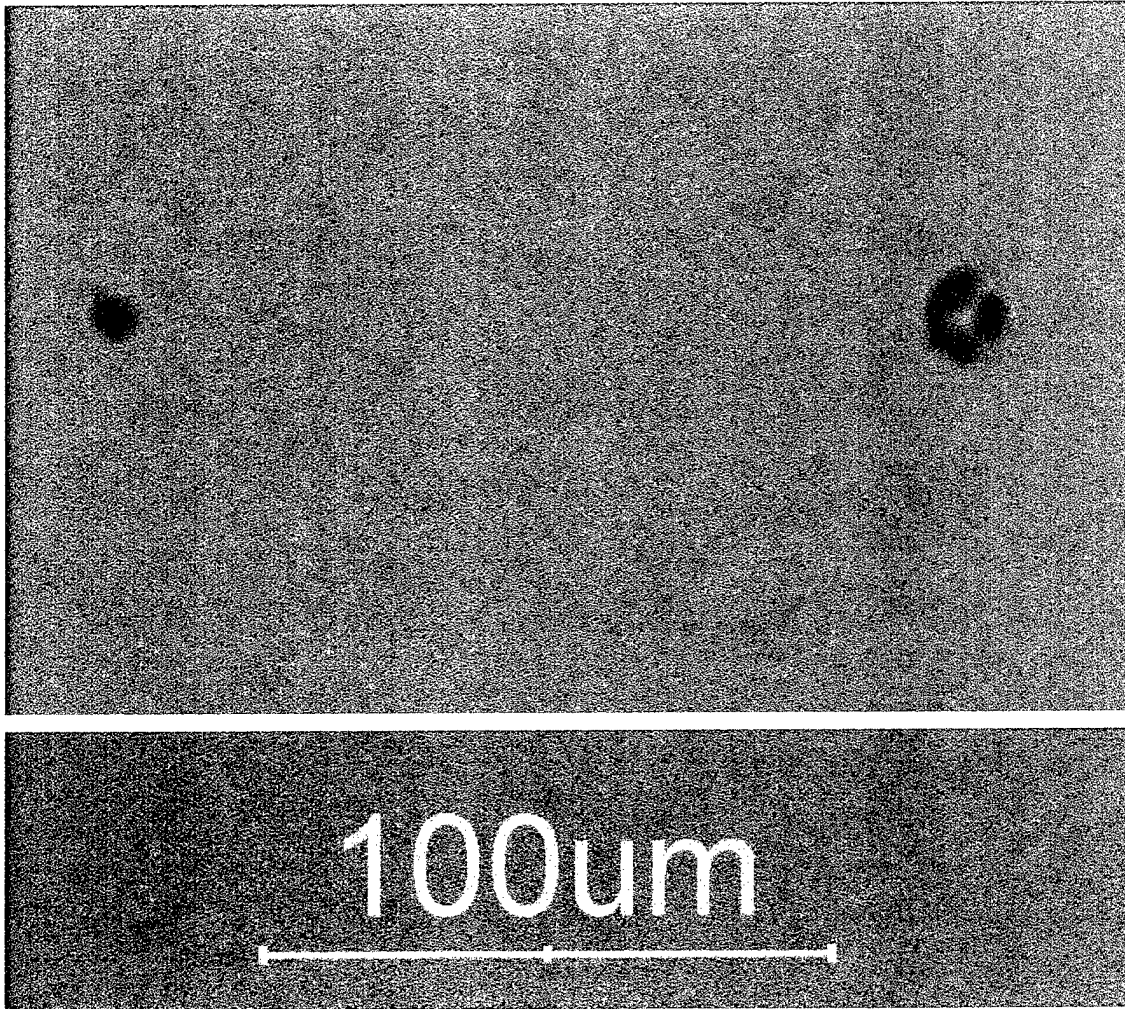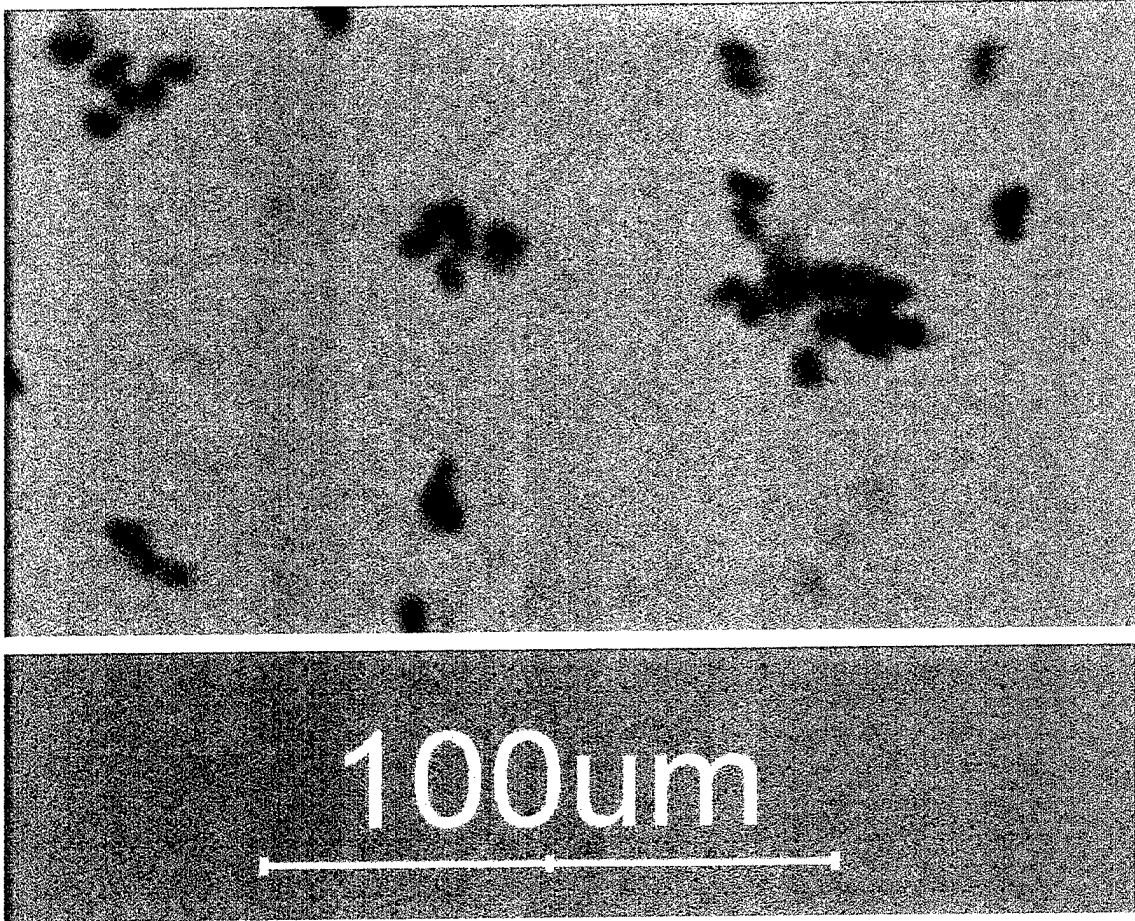
Figure 11. Spore stain of SIBCA sample 126

Figure 12. Spore stain of B. globigii spores

References

1. Thompson, H.G., Fulton, R.E., Fisher, G.R., and Stadnyk, L.L. *Light Addressable Potentiometric Immunoassays for Identification of Biological Agents: NATO SIBCA Exercise I,* DRES Technical Report 2001-035, 2001.

2. Fulton, R.E. and Stadnyk, L.L. *Immunochromatographic Assays for Identification of Biological Agents: NATO SIBCA Exercise I,* DRES Technical Report 2000-154, 2000.

3. Bader, D.E. and Fisher, G.R. *Molecular Genetic Analysis of Biological Warfare Agents: NATO SIBCA Exercise I,* DRES Technical Report 2000-188, 2000.

4. Bader, D.E. and Fisher, G.R. *Molecular Genetic Analysis of Biological Agents: NATO SIBCA Exercise II,* DRES Technical Report 2000-140, 2000.

5. Fulton, R.E. and Stadnyk, L.L. *Colorimetric Enzyme-Linked Immunosorbant Assays for Identification of Biological Agents: NATO SIBCA Exercise II,* DRES Technical Report 2000-111, 2000.

6. Bader, D.E., Fisher, G.R., and McLaws, L. *Molecular Genetic Analysis of Biological Agents: NATO SIBCA Exercise III,* DRDC Suffield Technical Report 2003-043, 2003.

7. Fulton, R.E. and Stadnyk, L.L. *Enzyme-Linked Immunosorbant Assays for Identification of Biological Agents in Sample Unknowns: NATO SIBCA Exercise III,* DRES Technical Report 2001-175, 2001.

8. Thompson, H.G., and Fulton, R.E., *Threshold^{TM} Immunoassays for Identification of Biological Agents: NATO SIBCA Exercise III,* DRDC Suffield Technical Report 2002-008, 2002.

9. Di Ninno, V. Scilab Consulting Inc. *Production of Goat and Rabbit Polyclonal Antibodies,* SSC File No. W7702-4-R430, Final Report, 30 March 1988.

10. Smith, R. Canadian Bioconcepts Inc. *Development of Antibodies for the Canadian Biochemical Detector Project: I. Bacterial Agents,* SSC File No. W7702-3-R362, Final Report, 1995.

11. Bader, D.E. and Fisher, G.R. *Molecular Genetic Analysis of Killed Biological Agents in Sample Unknowns: NATO SIBCA Exercise IV,* DRDC Suffield Technical Report 2003-112, 2003.

12. Lee, W.E, Thompson, H.G., and Kokko, E. *The Antigenicity of Bacillus globigii Spores,* DRES Memorandum 1500, 1998.

13. Fox, A., Black, G.E., Fox, K., and Rostovtseva, S. *Determination of Carbohydrate Profiles of Bacillus anthracis and Bacillus cereus Including Identification of O-methyl methylpentoses by Using Gas Chromatography-mass Spectrometry*, J. Clin. Microbiol. 31: 887-894, 1993.

14. Turnbull, P.C., Hutson, R.W., Ward, M.J., Jones, M.N., Quinn, C.P., Finnie, N.J., Duggleby, C.J., Kramer, J.M., and Melling, J. *Bacillus anthracis But Not Always Anthrax*, J. Appl. Bacterol. 72: 21-28, 1992.

15. Kaneko, T., Nozaki, R., and Aizawa, K. *Deoxyribonucleic acid Relatedness Between Bacillus anthracis, Bacillus cereus and Bacillus thuringiensis*, Microbiol. Immunol. 22: 639-641, 1978.

16. Ash, C., Farrow, J.A., Dorsch, M., Stackebrandt, E., and Collins, M.D. *Comparative Analysis of Bacillus anthracis, Bacillus cereus and Related Species on the Basis of Reverse Transcriptase Sequencing of 16s rRNA*, Int. J. Syst. Bacteriol. 41: 343-346, 1991.

17. Ash, C. and Collins M.D. *Comparative Analysis of 23s Ribosomal RNA Gene Sequences of Bacillus anthracis and Emetic Bacillus cereus Determined by PCR-direct Sequencing*, FEMS Microbiol. Lett. 94: 75-80, 1992.

18. Harmon, S.M. *New Method for Differentiating Members of the Bacillus cereus Group: Collaborative Study*, J. Assoc. Off. Anal. Chem. 65: 1134-1139, 1982.

19. Longchamp, P. and Leighton, T. *Molecular Recognition Specificity of Bacillus anthracis Spore Antibodies*, J. Appl. Microbiol. 87: 246-249, 1999.

20. Jackson, P.J., Hill, K.K., Laker, M.T., Ticknor, L.O. and Keim, P. *Genetic Comparison of Bacillus anthracis and its Close Relatives Using Amplified Fragment Length Polymorphism and Polymerase Chain Reaction Analysis*, J. Appl. Microbiol. 87: 263-269, 1999.

21. Phillips, A.P., Campbell, A.M. and Quinn, R. *Monoclonal Antibodies Against Spore Antigens of Bacillus anthracis*, FEMS Microbiol. Immunol. 47: 169-178, 1988.

22. Titball, R.W., Turnbull, P.C. and Hutson, R.A. *The Monitoring and Detection of Bacillus anthracis in the Environment.* J. Appl. Bacterol. Symposium Suppl. 70: 9S-18S, 1991.

23. Ezzell, J.W., Abshire, T.G., Little, S.F., Lidgerding, B.C., and Brown, C. *Identification of Bacillus anthracis by Using Monoclonal Antibody to Cell Wall Galactose-n-acetylglucosamine Polysaccharide*, J. Clin. Microbiol. 28: 223-231, 1990.

24. Phillips, A.P. and Martin, K.L. *Investigation of Spore Surface Antigens in the Genus Bacillus by the Use of Polyclonal Antibodies in Immunofluorescence tests*, J. Appl. Bacteriol. 64: 47-55, 1988.

25. Niederwohrmeier, B. and Bohm, R. *Enzyme Immunoassay for Rapid Detection of Bacillus anthracis*, Salisbury Medical Bull (Suppl.) 68: 24-25, 1990.

26. Phillips, A.P., Martin, K.L. and Broster, M.G. *Differentiation Between Spores of Bacillus anthracis and Bacillus cereus by a Quantitative Immunofluorescence Technique*, J Clin. Microbiol. 17: 41-47, 1983.

27. Phillips, A.P. and Ezzel, J.W. *Identification of Bacillus anthracis by Polyclonal Antibodies Against Extracted Vegetative Cell Antigens*, J. Appl. Bacteriol. 66: 419-432, 1989.

28. NATO International Staff-Defence Support Division *NATO Handbook for Sampling and Identification of Biological and Chemical Agents*, Vol. 1 (2000): Procedures and Techniques, AEP-10.

29. Harper, B. *NATO Land Group 7/SIBCA 4th International Laboratory Training Exercise on the Identification of Biological Warfare Agents (Killed)*, presented at meeting of Subgroup on Sampling and Identification of Chemical, Biological and Radiological Agents, Warsaw, Poland, 17-20 Nov., 2003.

DOCUMENT CONTROL DATA
(Security classification of title, body of abstract and indexing annotation must be entered when the overall document is classified)

1.	ORIGINATOR (the name and address of the organization preparing the document. Organizations for who the document was prepared, e.g. Establishment sponsoring a contractor's report, or tasking agency, are entered in Section 8.) Defence R&D Canada – Suffield	2.	SECURITY CLASSIFICATION (overall security classification of the document, including special warning terms if applicable) Unclassified

3.	TITLE (the complete document title as indicated on the title page. Its classification should be indicated by the appropriate abbreviation (S, C or U) in parentheses after the title). Enzyme-linked Immunosorbant Assays for Identification of Biological Agents in Sample Unknowns: NATO SIBCA Exercise IV (U)

4.	AUTHORS (Last name, first name, middle initial. If military, show rank, e.g. Doe, Maj. John E.) Thompson, H.G. and Fulton, R.E.

5.	DATE OF PUBLICATION (month and year of publication of document) December 2003	6a. NO. OF PAGES (total containing information, include Annexes, Appendices, etc) **49**	6b. NO. OF REFS (total cited in document) **29**

7.	DESCRIPTIVE NOTES (the category of the document, e.g. technical report, technical note or memorandum. If appropriate, enter the type of report, e.g. interim, progress, summary, annual or final. Give the inclusive dates when a specific reporting period is covered.) Technical Report

8.	SPONSORING ACTIVITY (the name of the department project office or laboratory sponsoring the research and development. Include the address.)

9a.	PROJECT OR GRANT NO. (If appropriate, the applicable research and development project or grant number under which the document was written. Please specify whether project or grant.)	9b.	CONTRACT NO. (If appropriate, the applicable number under which the document was written.)

10a.	ORIGINATOR'S DOCUMENT NUMBER (the official document number by which the document is identified by the originating activity. This number must be unique to this document.) DRDC Suffield TR 2003-120	10b.	OTHER DOCUMENT NOs. (Any other numbers which may be assigned this document either by the originator or by the sponsor.)

11.	DOCUMENT AVAILABILITY (any limitations on further dissemination of the document, other than those imposed by security classification) (x) Unlimited distribution ( ) Distribution limited to defence departments and defence contractors; further distribution only as approved ( ) Distribution limited to defence departments and Canadian defence contractors; further distribution only as approved ( ) Distribution limited to government departments and agencies; further distribution only as approved ( ) Distribution limited to defence departments; further distribution only as approved ( ) Other (please specify): No release without reference to DRDC Suffield DRP

12.	DOCUMENT ANNOUNCEMENT (any limitation to the bibliographic announcement of this document. This will normally corresponded to the Document Availability (11). However, where further distribution (beyond the audience specified in 11) is possible, a wider announcement audience may be selected).

13. ABSTRACT (a brief and factual summary of the document. It may also appear elsewhere in the body of the document itself. It is highly desirable that the abstract of classified documents be unclassified. Each paragraph of the abstract shall begin with an indication of the security classification of the information in the paragraph (unless the document itself is unclassified) represented as (S), (C) or (U). It is not necessary to include here abstracts in both official languages unless the text is bilingual).

In January 2002, the NATO Panel VII Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the fourth international training exercise on identification of biological agents. Fourteen NATO/Partners for Peace national laboratories participated: Austria, Bulgaria, Canada, Denmark, France, Germany (two laboratories), Italy, the Netherlands, Norway, Poland, Sweden, the United Kingdom, and the United States. The designated laboratory for Canada was Defence R&D Canada – Suffield (DRDC Suffield). Participant laboratories were sent six swabs. Participants were advised that samples would contain any one of the following gamma-irradiated organisms: *Bacillus anthracis, Yersinia pestis, Brucella melitensis, Francisella tularensis, Vibrio cholerae, Burkholderia mallei*, Venezuelan equine encephalitis (VEE) virus, vaccinia virus, *Coxiella burnetii*, or yellow fever virus. A number of immunologically-based technologies were used at DRDC Suffield for screening of sample unknowns, one of which was the enzyme-linked immunosorbant assay (ELISA). Antigen capture ELISAs were developed for all 10 possible biological agents and were used to screen the samples and a heterologous agent panel included as a control for specificity. Five biological agent unknowns were identified by ELISA, three at the species level: *B. melitensis, F. tularensis,* and *Y. pestis,* and two at the genus level: *Bacillus spp.* One sample containing *V. cholerae* produced a false negative reaction. A comparison of the ELISA results with the identity of organisms in SIBCA sample unknowns, as revealed by Dugway Proving Ground following the exercise, indicated correct identification of three of the samples, a partially correct identification of two samples, and an incorrect false negative for one sample.

14. KEYWORDS, DESCRIPTORS or IDENTIFIERS (technically meaningful terms or short phrases that characterize a document and could be helpful in cataloguing the document. They should be selected so that no security classification is required. Identifies, such as equipment model designation, trade name, military project code name, geographic location may also be included. If possible keywords should be selected from a published thesaurus, e.g. Thesaurus of Engineering and Scientific Terms (TEST) and that thesaurus-identified. If it is not possible to select indexing terms which are Unclassified, the classification of each should be indicated as with the title.)

Enzyme-Linked Immunosorbant Assay
ELISA
SIBCA
Exercise IV
Biological Agents
Threshold[TM]
Spore stain
Gram stain